Effector biology of the sugar beet pathogen *Cercospora beticola*

Malaika K. Ebert

Propositions

1. CbNip1 evolved to compensate for the lack of cercosporin functionality in the dark.
   (this thesis)

2. Microbial toxin auto-resistance mechanisms have the potential to provide durable resistance in crop plants.
   (this thesis)

3. After high-throughput genotyping, high-throughput phenotyping is the next major challenge plant science researchers have to overcome.

4. Integration of the knowledge on crop-associated microbiomes into farming techniques has the potential to improve farming.

5. Every job application deserves a reply.

6. Drones will revolutionize modern farming.

Propositions belonging to the thesis, entitled
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Thesis

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Chapter 1

General introduction and outline of the thesis

Introduction

Interactions between plant pathogenic fungi and their hosts are comprised of various, complex events that often occur simultaneously. In order to successfully establish disease, the fungus has to circumvent the plant innate immune system that relies on immune receptors to sense invading pathogens. The initial model to elucidate interactions between pathogen-derived ligands and plant immune receptors was formulated by Flor (1942) (1), and is better known as the "gene-for-gene hypothesis". It proposes that race-specific resistance in plants is determined by corresponding gene pairs, more precisely the products of resistance genes (*R genes*) in the plant are able to recognize the products of corresponding pathogen-derived avirulence genes (*Avrs*). A couple of years later, a new model was introduced, the so called "zigzag model". This model illustrates that general, non-race specific, elicitors also known as microbe-associated molecular patterns (MAMPs) can be perceived by the plant through pattern recognition receptors (PRRs) located on the cell surface which will elicit a first immune defense response named MAMP-triggered immunity (MTI) (2-7). In turn, pathogens have developed effectors to counteract MTI which leads to effector-triggered susceptibility (ETS). Detection of effectors by intracellular receptors encoded by *R* genes results in effector-triggered immunity (ETI) unless the pathogen is able to suppress (ETI) by loss or modification of the recognized effectors, or utilization of novel effectors.

With the introduction of the conceptual "Invasion Model", problems of the zigzag model were solved such as the depiction of MTI and ETI being displayed as being separated in time and space as well as the conceptual conflict that MAMPs are defined from the perspective of the plant while effectors are defined from the perspective of the invading microbe. The Invasion Model states that plants have evolved invasion pattern receptors (IPRs) to recognize microbial or modified-self ligands (termed invasion patterns (IPs) that may elicit an IP-triggered response (IPTR) with the intention to reveal invasion (8). Plant pathogens on the other hand can potentially suppress IPTRs by secreting effector proteins. An example for IP-IPR interaction can be found between the soil-borne pathogen *Verticillium dahliae*, that causes Verticillium wilt disease in over 200 dicotyledonous plant species (9, 10) and one of its host tomato. In tomato, the immune receptor Ve1 has been shown to mediate resistance to *V. dahliae* race 1 strains that secrete the effector VdAve1 (avirulence on Ve1 tomato) that is recognized as an IP (11). While it has been shown that VdAve1 perception activates Ve1-mediated resistance in tomato, Ave1 was also found to play an important role in disease, as *Ave1* deficient *V. dahliae* mutants are hampered in virulence on tomato lines lacking Ve1 and on Arabidopsis (12).

Interestingly, successful IPTR signaling of the host, does not necessarily lead to the abolishment of infection (8). In fact, some plant pathogenic fungi deliberately evoke IPTRs in their hosts. For example, the necrotrophic fungus *Parastagonospora nodorum*, a wheat pathogen that causes Septoria nodorum blotch, has mastered this approach by recruiting IP-IPR interactions for cell death induction to establish disease (13, 14). Nine interactions are currently known between *P. nodorum* secreted IPs and corresponding wheat susceptibility genes/ IPRs (13, 15-28). Although fungi like *P. nodorum* appear to deliberately hijack IPTR to trigger cell death in the host, some pathogen-derived effectors can cause plant cell death solely due to their toxic nature. For

example, a protein family with such a cytotoxic character are the Nep1-like proteins (NLPs) (29). Here, toxicity is hypothesized to be linked to cytolytic activity of the proteins resulting in plant plasma membrane depolarization and, subsequently, cell death (30-33). The wheat pathogen *Zymoseptoria tritici*, a hemibiotrophic Dothideomycete that causes septoria tritici blotch, for example harbors MgNLP whose product is able to cause cell death (34). However, MgNLP necrosis-inducing activity was characterized as selective as necrosis formation was observed in Arabidopsis and tobacco but not in wheat.

Besides effector proteins, secondary metabolites (SMs) are also well-known for their toxic properties. For instance, *Alternaria* spp. are omnipresent saprophytic or pathogenic fungi with a broad host range and are notorious producers of a variety of host-specific toxins that display diverse modes of action (35, 36). For example, the host-specific *Alternaria* toxins ACT-toxin, AF-toxin, and AK-toxin have a 9,10-epoxy-8-hydroxy-9-methyl-decatrienoic acid (EDA) backbone and are therefore members of the EDA family that have the potential to modify the plasma membrane of susceptible host cells (37-40). AK toxin I of the *Alternaria alternata* Japanese pear pathotype has been shown to induce plasma membrane modifications such as plasmalemmal invaginations, fusion of Golgi vesicles to invaginated plasma membranes, and accumulation of polysaccharides and membrane fragments derived from invaginated plasma membranes in susceptible pear plants (41-44). Associated with membrane modification was the formation of reactive oxygen species (ROS) that are hypothesized to be responsible for lipid peroxidation (42). Furthermore, irreversible depolarization of the plasma membrane results in subsequent electrolyte leakage which ultimately leads to host cell death (35, 39, 40, 44, 45).

In contrast to host-specific toxins that require a corresponding molecular target to be present in their host in order to be effective, some fungi also produce toxins, termed non host-specific toxins that are almost universally toxic. For example, toxins belonging to the family of perylenequinones are considered to be non host-specific because they cause cell damage nearly universally to living cells (46-51). One of the most well-studied members of the perylenequinone family is cercosporin. Species in the genus *Cercospora* have been shown to rely on cercosporin to facilitate infection (52-54). As for all other perylenequinones, toxicity of this molecule can be traced back to the 3,10-dihydroxy-4,9-perylenequinone backbone, a common feature among all members of the perylenequinone family (Figure 1) (55-57). This chromophoric core allows absorption of light in the range of visible to UV light, leading to energetic excitation of the compound. Once cercosporin reaches an excited triplet state it reacts with oxygen leading to the formation of ROS. The emerging ROS have the capability to induce cell death of the host by causing lipid peroxidation and indiscriminate damage to proteins and DNA (58, 59). While cercosporin production has been demonstrated for *Cercospora* species and recently also for *Colletorichum fioriniae* (60), many other plant pathogenic fungi such as *Cladosporium phlei*, causal agent of leaf spot disease of timothy (61), or the citrus pathogen *Elsinoë fawcettii* (62) have been shown to produce structurally similar perylenequinones that share the same mode of action.

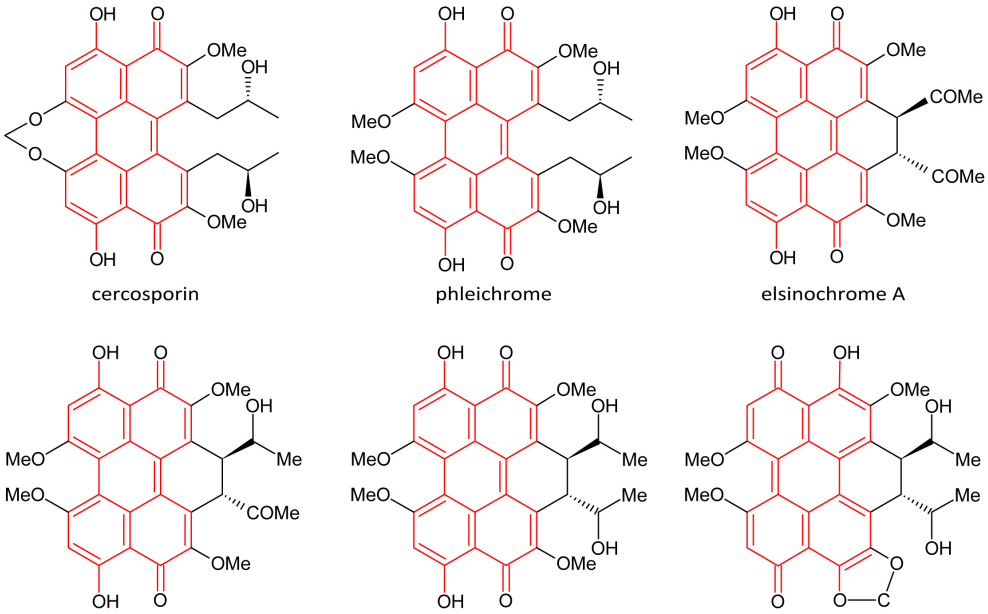

Figure 1. Structures of related perylenequinones. Cercosporin secreted by *C. beticola*, phleichrome by *C. phlei* and elsinochromes A, B, C, and D produced by *E. fawcettii* are structurally related as they share a common backbone (indicated in red). Structural differences between the molecules are mostly due to various side chains.

Cercospora beticola – my pathogen of interest

Cercospora beticola is a hemibiotrophic fungus that causes Cercospora leaf spot disease on sugar beet plant (*Beta vulgaris*), the most destructive foliar disease on sugar beet worldwide (63). In the field, *C. beticola* can over-winter as stromata in infected leftover leaf material until conidiospores are disseminated throughout the field by insects, wind, and water splash (63-65). On the sugar beet leaf, germinating spores enter the host through stomata to gain access to the apoplast where the fungus continues growing intercellularly (63, 66). While the initial phase of colonization is asymptomatic, small, circular necrotic spots will form when the fungus switches to its necrotrophic phase (63, 66, 67). The increase in lesion number causes the lesions to fuse which leads to large necrotic tissue patches on the leaf where new conidia are formed. These conidia can again serve as inoculum for a new disease cycle. The utilization of effectors to facilitate disease has been demonstrated for many pathogenic fungi. However, the only *C. beticola* effector identified to-date is the secondary metabolite cercosporin. The ability to produce cercosporin is conserved in almost all *Cercospora* species and targeted gene replacement studies revealed that cercosporin contributes to virulence in multiple *Cercospora* species (48, 52-54).

Thesis outline

In this thesis, the effector repertoire of the fungal sugar beet pathogen *Cercospora beticola* was investigated. Additionally, I extended my studies to other fungal pathogens that have been

identified to produce toxins that are structurally-related and therefore belong to the same toxin family as to cercosporin produced by *C. beticola*.

Plant pathogenic fungi utilize effectors to promote virulence during colonization of the host plant. These effectors often have diverse modes of action and can be derived from proteins or secondary metabolites. **Chapter 2** gives an overview of the broad diversity of known fungal virulence mechanisms.

The effector protein VdAve1 (*Verticillium dahliae* Avirulence on Ve1 tomato) was originally identified as a race 1 specific effector protein of the soil-borne pathogen *Verticillium dahliae*. In tomato, resistance to *V. dahliae* race 1 strains is conferred by the extracellular leucine-rich repeat cell surface receptor Ve1. While homologs of *VdAve1* can primarily be found in plants, some plant pathogenic fungi also harbor homologs of *VdAve1* such as *Fusarium oxysporum (FoAve1), Colletotrichum higginsianum (ChAve1)* and *Cercospora beticola (CbAve1)*. In **Chapter 3**, we determined the gene expression profiles of CbAve1, FoAve1, and ChAve1 during host infection by the producing pathogen and examined whether these genes are necessary for full virulence of their producer.

As a hemibiotrophic fungus, *C. beticola* relies on host cell death to provide nutrients during the necrotrophic stage of the lifecycle. Therefore, we hypothesized that the fungus secretes effector proteins during infection that facilitate disease by causing necrosis formation. **Chapter 4** describes a phenotype-based approach that aimed to identify conditions in which the fungus produces effector proteins *in vitro*. The expression profile of the effector candidate CbNip1 during *C. beticola* infection was determined and the candidate was heterologously expressed in *E. coli* for functional analysis. Furthermore, contribution of the candidate to *C. beticola* virulence was assessed.

Apart from proteinaceous effectors *C. beticola* is a well-known producer of phytotoxic secondary metabolites. During infection, *C. beticola* produces the perylenequinone cercosporin. This secondary metabolite is formed via a polyketide synthesis gene cluster that for decades was thought to consist of eight genes. However, **Chapter 5** describes the identification of additional cercosporin biosynthesis genes. Targeted gene replacement of novel cercosporin biosynthesis genes aimed to determine their involvement in toxin formation. Furthermore, orthologous cercosporin clusters in other Ascomycetes were identified through phylogenetic analysis.

Due to high structural similarity of perylenequinone family members, it was hypothesized that the biosynthetic gene clusters responsible for perylenequinone formation display considerable similarity between species. **Chapter 6** utilizes gene cluster conservation to facilitate the identification of perylenequinone and DHN-melanin biosynthesis pathways in multiple fungal species.

Finally, **Chapter 7** discusses the major findings described in this thesis and provides an overview of the effector repertoire of *C. beticola* at its current state.

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Chapter 2

Tools of the crook – infection strategies of fungal plant pathogens

Luis Rodriguez-Moreno*, Malaika K. Ebert*, Melvin D. Bolton#, and Bart P.H.J. Thomma# (2018) Tools of the crook – infection strategies of fungal plant pathogens. *The Plant Journal* 93, 664–674; doi: 10.1111/tpj.13810

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Abstract

Fungi represent an ecologically diverse group of microorganisms that includes plant pathogenic species able to cause considerable yield loses in crop production systems worldwide. In order to establish compatible interactions with their hosts, pathogenic fungi rely on the secretion of molecules of diverse nature during host colonization to modulate host physiology, manipulate other environmental factors or provide self-defence. These molecules, collectively known as effectors, are typically small secreted cysteine-rich proteins, but may also comprise secondary metabolites and sRNAs. Here, we discuss the most common strategies that fungal plant pathogens employ to subvert their host plants in order to successfully complete their life cycle and secure the release of abundant viable progeny.

Introduction

Fungi constitute an evolutionarily and ecologically diverse group of microorganisms that includes plant pathogenic species that cause considerable yield losses in agricultural production systems worldwide. Generally, the lifestyles of plant pathogenic fungi are differentiated depending on the strategies used to acquire nutrients from their hosts. As such, obligate biotrophic fungi comprise those species that can only feed on living host tissue to meet their nutritional requirements and complete their life cycle. At the complete opposite of the spectrum, necrotrophic fungi trigger cell death in the host to secure nutrient supply. In between these extremes is a wide array of hemibiotrophic fungi that start their compatible host interaction with an initial biotrophic phase that, at one point in time when the infection progressed sufficiently, is followed by a transition to a necrotrophic stage. A parasitic life style that involves the extraction of sugars from other organisms is one of the ways in which non-heterotrophic organisms compensate for the inability to generate sugars through photosynthesis. Many biotrophic and hemibiotrophic fungi evolved haustoria, appendages of fungal hyphae that invaginate the host plasma membrane and grow inside host cells, to obtain these nutrients. Recently, it was demonstrated that the obligate biotrophic powdery mildew fungus *Golovinomyces cichoracerum* requires lipids for colonization that it receives from the host plant (1).

Lifestyle differences largely determine the wide array of strategies that fungi use to evade, counteract or hijack plant defences in their effort to complete their life cycle and secure the production of viable progeny. Irrespective of their life style, microbial pathogens are all believed to utilize so-called effectors, in *planta*-secreted molecules of various nature, to support host colonization, often, but not exclusively, through suppression of host immune responses (2). Over the years it has become evident that haustoria are not only fungal feeding structures, but are also active sites for secretion and translocation of effectors into the host (3-6).

Plants have developed an innate immune system to recognize and respond to microbes (7-9) (Figure 1). This immune system relies on the presence of immune receptors that detect pathogen invasion through sensing of pathogen(-induced) ligands, collectively termed invasion patterns, to mount appropriate immune responses (9). Recognition of invasion patterns triggers both local and systemic reactions to respond in a quick and focussed manner to attempted microbial ingress (7-9). For example, the well-characterized invasion pattern chitin, an important constituent of fungal cell walls, is recognized by plants through plasma membrane-localized extracellular lysin motif (LysM)-containing receptor molecules (10, 11). Pathogen recognition by plant immune receptors causes ion fluxes, the accumulation of reactive oxygen species (ROS), and a quick activation of defence-related mitogen-activated protein kinase (MAPKs) cascades that cause an extensive transcriptional reprogramming of the host (12-14). Furthermore, pathogen perception leads to reinforcement of plant cell walls by callose deposition, changes in hormone biosynthesis, and the production of antimicrobial compounds (15). In many cases, these defence responses collectively are sufficient to render the interaction between the plant and the invader incompatible, implying that pathogen ingress is halted or at least significantly slowed down. However, co-evolutionary processes have selected pathogens that employ a plethora of virulence strategies to overcome various mechanisms within plant immune systems.

In this review, we summarize the different virulence strategies that plant pathogenic fungi use to subvert their hosts. While there are excellent reviews that discuss individual strategies in detail, the aim of this review is to outline the broad diversity of known fungal virulence mechanisms (Figure 2).

Fungal strategies for host penetration

One of the first barriers that fungal pathogens have to breach to gain entrance to their hosts is cell walls that are mainly composed of carbohydrates. Many plant pathogenic fungi utilize specialized infection structures, called appressoria, to generate focused turgor pressure to breach the cell wall by force (16). Depending on the fungal species, the turgor pressure is combined with the localized release of cell wall-degrading enzymes (CWDEs) (16). Furthermore, effectors are secreted from appressorial penetration pores prior to host invasion (17).

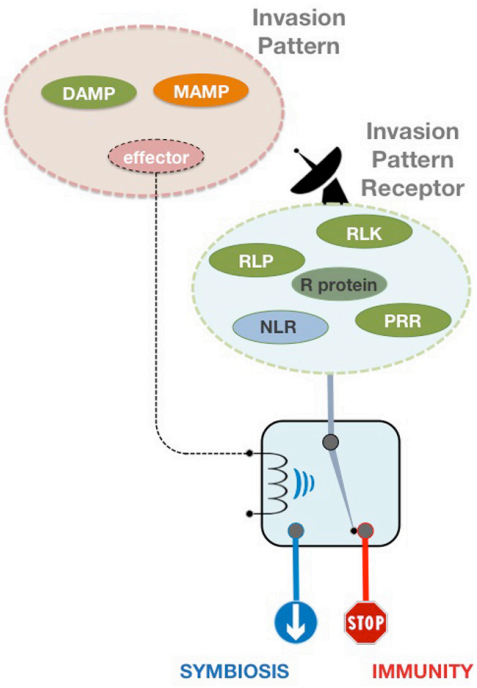

Figure 1. Schematic representation of the "Invasion Model" to describe the molecular basis of plant immunity against fungal pathogens. In this model invasion pattern receptors, comprising any type of host receptor, detect invasion patterns, comprising externally encoded and modified-self ligands that announce invasion, to mount an effective immune response and halt the symbiosis. Fungal effectors may manipulate the induced response to tweak the symbiosis to their benefit.

Fungi typically produce an arsenal of so-called carbohydrate-active enzymes (CAZymes) that are grouped into five enzyme classes, namely glycoside hydrolases, glycosyltransferases, polysaccharide lyases, carbohydrate esterases, and redox enzymes with auxiliary activities (18). Several of the polysaccharide lyases, glycoside hydrolases, and carbohydrate esterases are known as CWDEs that are used to degrade host cell walls. Typically plant pathogenic species contain higher numbers of CAZyme genes than saprophytic and animal pathogenic strains (19). Whereas obligate biotrophs typically lack extensive catalogs of CWDE genes and likely only use such enzymes for subtle manipulations of host cell walls such as at the cellular entrance sites for haustoria, necrotrophic fungi were often thought of as 'brute-force' pathogens that rely on large CWDE catalogs to macerate host cell walls and initiate colonization (20). These enzymes occur in multiple isoforms that not only differ in isoelectric point and molecular weight, but also in timing of their production and processing, offering especially broad host-range necrotrophs particular flexibility to penetrate and colonize their hosts. Besides colonization, these enzymes also liberate nutrients for the pathogen. For example,

hydrolysis of pectin by fungal pectinases weakens the cell wall to enable penetration while also providing the fungus with important carbon sources for growth (21). Indeed, strategies to limit pectin degradation were explored by generating transgenic wheat lines expressing pectin methyl esterase inhibitors, which exhibited altered pectin methyl esterification that resulted in reduced activity of pathogen pectic enzymes and reduced disease from hemibiotrophic pathogens *Fusarium graminearum* and *Bipolaris sorokiniana* (22). Similarly, wheat lines expressing genes encoding a xylanase inhibitor and polygalacturonase inhibiting protein exhibited increased resistance to Fusarium head blight (23). However, *F. graminearum* single gene deletion mutants for polygalacturonase or xylanase resulted in minor effects on virulence, while double gene mutants were significantly reduced in virulence on soybean and wheat plants, highlighting the synergism between CWDEs (24).

Besides plant cell wall-degrading enzymes, fungi secrete CWDEs to modulate their own cell walls and accommodate morphological changes. It was recently proposed that such activity facilitates pathogenesis of plants by enabling host colonization. A glycosyltransferase enzyme from the hemibiotrophic wheat pathogen *Zymoseptoria tritici* was reported to enable hyphal growth on solid surfaces that is essential for fungal disease of wheat plants (25). Homologs of this particular enzyme are widespread in fungi, and mutants in the taxonomically unrelated *F. graminearum* were similarly impaired in hyphal growth and pathogenicity (25).

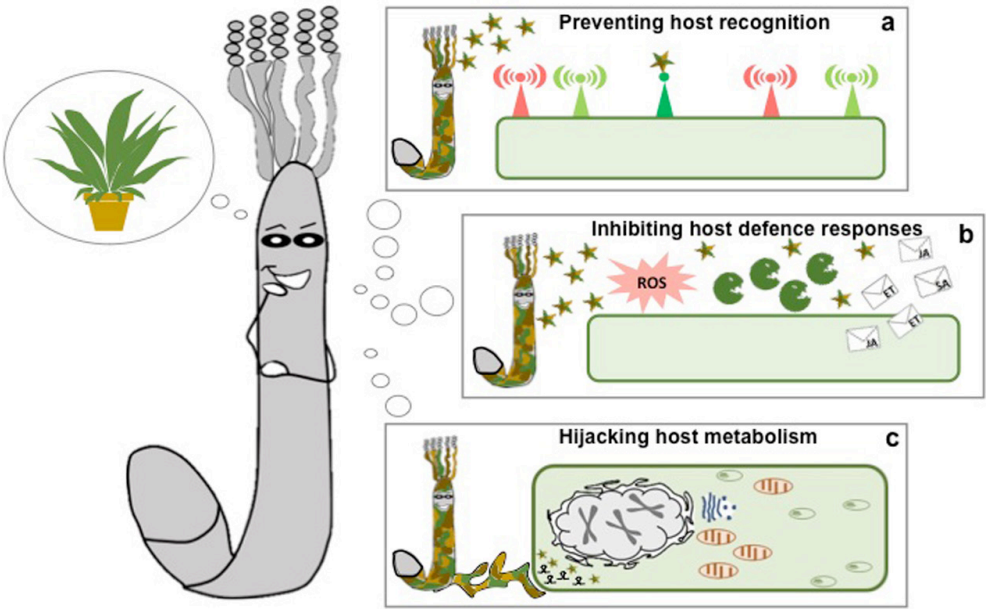

Figure 2. Illustration of fungal pathogen strategies to surmount host plants. (a) Secretion of effectors that perturb recognition by plant immune receptors. (b) Secretion of effectors that subvert plant defense responses that are induced upon pathogen detection. (c) Delivery of various types of molecules to hijack host metabolism.

Fungal strategies preventing plant recognition

Plants evolved a plethora of plasma membrane-localized immune receptors for surveillance of the extracellular space for pathogen(-induced) ligands (13, 15, 26). The perception of these ligands is relayed into downstream signalling events which lead to the activation of plant defences (14, 26). Structural components of the fungal cell wall, such as glucans and chitin, are typically recognised as pathogen ligands by plant receptors (10, 11, 27, 28). As part of their defence system, plants secrete glucanases and chitinases to compromise the integrity of fungal cell walls and release oligomeric fragments that can act as ligand for extracellular immune receptors (29). Fungi have evolved several strategies to overcome host immune responses that involve fungal cell walls, including alterations in cell wall compositions and the secretion of effectors to protect cell walls or perturb recognition of cell wall components (2, 29).

Magnaporthe oryzae is a hemibiotrophic fungal pathogen and causal agent of rice blast disease (30). During infection, *M. oryzae* responds to the epidermal wax component 1,16-hexadecanediol by accumulating α-1,3-glucans at the surface of the cell wall, resulting in inhibition of chitin degradation by plant chitinases (31). Accordingly, mutants that are unable to accumulate α-1,3-glucans at the fungal cell surface trigger rapid activation of host defences (31). A similar strategy has been reported for the maize pathogen *Colletotrichum graminicol*a that modifies the β-glucan composition of its biotrophic hyphae, as the content of β-1,3- and β-1,6-glucans is significantly reduced when compared with appressoria and necrotrophic hyphae (32-34). Hence, *C. graminicola* strains that overexpress a β-1,3-glucan synthase in their biotrophic hyphae induce stronger host defense responses and display reduced virulence (32). However, *C. graminicola* strains that are unable to produce β-1,6-glucans are defective in appressorium formation and thus non-pathogenic (34).

Modification of cell walls is not the only strategy employed by fungal pathogens to prevent plant recognition. For instance, the tomato leaf mould fungus *Cladosporium fulvum* secretes the carbohydrate-binding effector protein Ecp6 that suppresses chitin-triggered host immunity. The chitin-binding capacity of Ecp6 is mediated by three Lysin motifs (LysMs) (35, 36) that occur in proteins of a wide range of organisms to confer the ability to bind various types of polysaccharides, including peptidoglycan and chitin, through a conserved βααβ-fold (37). Interestingly, two out of the three LysM domains of Ecp6 cooperate to form a groove that binds chitin fragments with ultra-high (pM) affinity that allows to outcompete host receptors for chitin binding (36). Besides Ecp6, *C. fulvum* also secretes the chitin binding effector molecule Avr4 during host colonization. As opposed to LysMs, Avr4 binds chitin through an invertebrate chitin-binding module to protect the cell wall against hydrolysis by host enzymes (38, 39). In contrast to Avr4 homologs that only occur in a limited set of fungi that are closely related to *C. fulvum* (40), LysM effector proteins occur in a wide variety of fungi (41), and have been shown to suppress chitin-triggered immunity on various plant hosts such as for *Z. tritici* on wheat (42), for *M. oryzae* on rice (43), for *C. higginsianum* on Arabidopsis (44) and for *V. dahliae* on tomato (45).

Whereas chitin perception in plants is relatively well-understood (46), β-glucan perception and signalling mechanisms remain poorly characterized (47). The root endophyte *Piriformospora*

indica secretes the β-glucan-binding lectin effector FGB1 that suppresses β-glucan-triggered host immunity (48). Prevention of β-glucan detection by the plant seems important for successful fungal infection as overexpression of the *Piriformospora indica* FGB1 homolog in *Ustilago maydis* was shown to lead to an increase in virulence. Interestingly, FGB1 homologs are widespread in fungi (48).

A further strategy to protect fungal cell walls and prevent detection of cell wall components is through the secretion of proteases that affect hydrolytic host enzymes (49). Fungal chitinase-modifying proteins (CMPs) have been reported in several maize pathogens including *Bipolaris zeicola* (50), *Stenocarpella maydis* (51) and *Fusarium verticilloides* (52). Similarly, *F. oxysporum* f. sp. *lycopersici*, *Verticillium dahliae* and *Botrytis cinerea* were found to secrete CMPs that can degrade extracellular tomato chitinases (53).

Fungal strategies for inhibiting host defence responses

Pathogen recognition by plants results in a panoply of defence responses to hamper pathogen invasion. These responses comprise swift ion fluxes, pH changes, production of reactive oxygen species (ROS), but also the production of local and systemic signalling molecules and of antimicrobial compounds. Various mechanisms are employed by fungal pathogens to subvert such responses.

Subverting ROS damage

ROS production is mostly due to the activity of membrane bound NADPH-oxidases and cell-wall associated peroxidases (POX) (54-56). While relatively low concentrations of ROS have been reported to act as defence signalling molecules, (57-59), high concentrations of ROS are extremely harmful to cells as they have been shown to cause oxidative damage (60, 61). The apoplastic effector Pep1 of the biotrophic maize pathogen *Ustilago maydis* accumulates at sites where biotrophic hyphae move from cell to cell in maize tissue to inhibit the oxidative burst through inhibition of POX12, a type-III class heme-peroxidase that is highly induced after *U. maydis* penetration (62, 63). Pep1 only causes partial inhibition of the maize apoplastic peroxidase activity, suggesting that not all peroxidase-producing enzymes in the maize apoplast are targeted by Pep1 (63).

Manipulating tissue pH

Many fungal pathogens induce a pH shift in the host tissue surrounding the infection site (64). For instance, *S. sclerotiorum* causes acidification of the infection area through the production of oxalic acid, leading to rapid death of host tissues (20). However, other pathogens induce alkalinisation of host tissue (65, 66). During host colonisation, the vascular wilt pathogen *F. oxysporum* causes an increase of the extracellular pH from about 5 to 7 through the secretion of a peptide with homology to plant rapid alkalinizing factors (RALFs) (66, 67). Interestingly, *F. oxysporum* strains that are no longer able to produce this peptide trigger enhanced host defence, indicating a role in suppression of host immunity. Although this role in virulence has been challenged (68), RALF-encoding genes can be found in many fungal pathogens, suggesting a universal mechanism to alkalinize infection sites to suppress host immunity (66, 68).

Inhibition of host proteases

Many of the molecules that fungal pathogens secrete in order to establish the parasitic interaction with their hosts are of proteinaceous nature, and hence plants secrete proteases to undermine this pathogen strategy (69, 70). The apoplast of tomato and Arabidopsis contains various proteases that contribute to host defence (71, 72). Among these, the tomato apoplast contains the extracellular cysteine protease Rcr3 that plays a central role in resistance mediated by the Cf-2 immune receptor of tomato and that is activated by the *C. fulvum* effector Avr2 (73). The Avr2 effector inhibits the activity of Rcr3, likely causing a conformational change in the Rcr3 structure that is recognized by Cf-2 (73, 74). Besides Rcr3, Avr2 inhibits various other host proteases that are required for pathogen defence (71). Other fungal pathogens also produce protease effectors to inhibit host proteases, such as the *U. maydis* Pit2 effector (75, 76).

Subverting hormone signalling

Plant growth and their responses to environmental cues, including pathogens, are largely governed by phytohormones. Typically, salicylic acid (SA) signalling governs resistance against biotrophic pathogens whereas a combination of jasmonic acid (JA) and ethylene (ET) signalling activates resistance against necrotrophic pathogens (8, 77, 78). To a large extent, these signalling pathways act antagonistically and their balance needs to be governed carefully. Thus it is not surprising that pathogens evolved various strategies to affect phytohormone signalling. For instance, *U. maydis* secretes the chorismate mutase Cmu1 into host cells to perturb SA production by affecting the production of its precursor (79). Likely, Cmu1 acts in combination with the maize chorismate mutase Cm1 to increase the flow of chorismate from the plastid to the cytosol to diminish the available substrate for salicylic acid biosynthesis in plastids in turn (79). Furthermore, *U. maydis* produces Shy1, a salicylate hydroxylase that degrades SA during host invasion (80). Together these results suggest that perturbation of SA-mediated immunity is crucial for *U. maydis* colonization. Chorismate mutases have been identified in many eukaryotic plant pathogens pointing towards a common strategy for host manipulation. Similar to *U. maydis*, also *V. dahliae* has been proposed to target SA biosynthesis by secreting effectors with isochorismatase activity to hydrolyse isochorismate (81). Besides targeting SA signalling, fungal effectors that target JA signalling or ET signalling have been described as well (82, 83). For instance, the beneficial fungus *Laccaria* bicolor produces the Mycorrhiza-induced small secreted protein-7 (MiSSP7) during the interaction with its host *Populus trichocarpa* (83). Intriguingly, MiSSP7 interacts with the plant JASMONATE ZIM-DOMAIN (JAZ)-6 protein to provoke blockage of the expression of JA-inducible genes in the host to promote fungal colonization (83).

Besides the capacity to manipulate hormone balances in plant tissues, particular fungi appear have the ability to produce hormone-mimicking compounds to promote host colonization (84-87). For instance, *Fusarium pseudograminearum* produces cytokinin-like molecules that activate plant cytokinin signaling to reprogram the host (88).

The molecules that do the job: fungal effectors

Typically, fungal effectors are described as small secreted, cysteine-rich proteins that are produced during host invasion (89). These fungal effectors can be divided into two types based on their

extra- or intracellular localization in the host. Yet, how cytoplasmic effectors are translocated into host cells remains poorly understood (90-92). Nevertheless, two distinct secretion systems to target effectors have been described for *M. oryzae*. Cytoplasmic effectors accumulate in a so-called biotrophic interfacial complex, a plant membrane-rich structure associated with invasive hyphae that involves exocyst and t-SNARE components (93, 94). By contrast, apoplastic effectors are secreted from invasive hyphae via conventional secretion. In addition to proteinaceous effector molecules, other types of molecules are secreted by fungi with the aim to establish the parasitic relationship that therefore qualify to be labelled as effectors just as well.

Secondary metabolites

Secondary metabolites (SMs) are small bioactive molecules that often play crucial roles in the establishment of specific ecological niches but, unlike primary metabolites, are not essential for fungal growth, development, or reproduction. While fungal SMs are often known and valued for their anti-microbial activities, many fungi employ SMs to promote virulence. Traditionally SMs involved with virulence are classified as either host-specific toxins (HSTs; discussed below), because they have specific targets in the host, or non-HSTs that typically do not have a specific host target and are generally toxic to a wide-range of organisms including the host instead (95). Perylenequinones, for example, are a family of photosensitizing SMs for which the mode of action is well-studied. The most prominent member of the family is cercosporin. This light-activated toxin is produced by most *Cercospora* spp. and has a very broad toxicity range to many organisms including plants, animals, bacteria and most fungi. Due to its photosensitizing nature, cercosporin is able to absorb light energy and subsequently react with oxygen (96). Products of this reaction are ROS that can cause protein and DNA damage and lipid peroxidation and eventually lead to cell death of the host (97, 98). As necrosis development lays the ground for fungal spore formation, it is speculated that cercosporin secretion might facilitate cell wall breaching to enable conidiophore and conidia production (99). The cercosporin biosynthesis gene cluster was recently shown to be found wide-spread in the *Colletotrichum* genus, implicating the role of cercosporin as a virulence factor in an important group of fungal plant pathogens (100).

Host-selective toxins

HSTs are known to induce necrotic host tissue reactions to promote host susceptibility (95). The effectiveness of HSTs depends on whether a plant possesses a corresponding toxin target, which may also define the host range of the producing pathogen. For example, maize lines harboring Texas cytoplasm for male sterility (Tcms) display extreme sensitivity to T-toxin and PM-toxin secreted by *Cochliobolus heterostrophus* race T and *Mycosphaerella zeae-maydis*, respectively (95, 101, 102). Here, host susceptibility is conferred by a single plant gene *T-urf13* that encodes URF13, a mitochondrial membrane protein to which either toxin can directly bind. Binding triggers URF13 to experience a conformational change which in turn results in the formation of a pore in the mitochondrial membrane. The ability to produce T-toxin is relevant for fungal virulence, as *C. heterostrophus* race O, a natural T-toxin lacking race and Tox1- deficient mutants of race T show reduced virulence on Tcms carrying maize (103). Similarly, PM-toxin deficient Tox- mutants of *M. zeae-maydis* lost the ability to infect Tcms maize (104). Besides mitochondria,

HSTs are also reported to target enzymes or other plant cell organelles like plasma membrane, chloroplast, endoplasmatic reticulum, nucleus, vacuole, and Golgi bodies with the objective to suppress host defense responses and/or induce host cell death (105).

Another HST toxin is victorin, a family of related, cyclized pentapeptides (106, 107) secreted by the necrotrophic fungus *Cochliobolus victoriae* that causes Victoria blight on susceptible oats (108). Fungal pathogenicity is solely attributed to the ability to produce victorin, as victorin deficient mutants are entirely non-pathogenic (109). While susceptibility of oats can be traced back to one dominant gene called *Vb* (110), it was later found that a single, dominant gene, called Locus Orchestrating Victorin Effects1 (LOV1), provides victorin susceptibility in Arabidopsis plants (111). Interestingly, only oat lines carrying Pc2, a resistance gene against crown rust, are susceptible to victorin producing *C. victoriae* isolates (110, 112). As studies to create plants resistant to both Victoria blight and crown rust were unsuccessful, it was suggested that *Vb* and *Pc2* are the same gene conferring susceptibility and resistance respectively (112, 113). Further evidence for this hypothesis was provided by the discovery that victorin perception triggers a defence response in susceptible oats and Arabidopsis (95, 111) hinting that *C. victoriae* hijacks the classic gene-for-gene interaction needed to provide resistance against crown rust and utilizes victorin to elicit host cell death via the same defense mechanism to suit its necrotrophic lifestyle.

The necrotrophic fungus *Parastagonospora nodorum* (formerly *Stagonospora nodorum*) is the causal agent of the Septoria nodorum blotch (SNB) disease on wheat (114, 115). Besides the ability to produce CWDEs and nonspecific toxins, *P. nodorum* has been characterized for its ability to produce a wide range of HSTs (also called necrotrophic effectors) that result in different levels of susceptibility depending on the wheat cultivar (116-119). So far, a total of nine interactions between necrotrophic effectors of *P. nodorum* and corresponding wheat susceptibility genes have been found (120-133). Furthermore, it was reported that homologs of the necrotrophic *P. nodorum* effector gene *ToxA* have been acquired via horizontal gene transfer and interspecific hybridization by the wheat pathogens *P. tritici-repentis, Phaeosphaeria avenaria triti,* and *Bipolaris sorokiniana* (134). So far, two host targets *Snn1* and *Tsn1*, of *P. nodorum* necrotrophic effectors ToxA and Tox1, respectively, have been cloned (135, 136). While *Tsn1* resembles a plant resistance gene structure as it harbors a serine/threonine protein kinase, a nucleotide binding, and leucine-rich repeat domains (136), *Snn1* is a wall associated kinase with a predicted transmembrane domain (135). However, in both cases interaction with a corresponding necrotrophic effector leads to a so called necrotrophic effector-triggered susceptibility (124, 135-137) in opposition to the conventional effector-trigger immunity (ETI) observed in most of the biotrophic interactions. Taken together, these studies suggest that some necrotrophic fungal pathogens use effectors to subvert the host resistance mechanism for their own benefit (95, 137, 138).

Non-typical effectors: sRNAs

Small RNAs (sRNA) induce gene silencing by binding to Argonaute (AGO) proteins and directing the RNA-induced silencing complex (RISC) to genes with complementary sequences (139). As regulatory molecules, sRNAs are involved in a wide range of biological processes such as organ morphogenesis, genome modification, and adaptive responses to abiotic and

biotic stresses (140-142). Both animals and plants have been reported to exchange sRNAs with parasites, pathogens, or symbiotic organisms in cross-kingdom sRNAs transfer (143). It is generally assumed that sRNAs from plants are integral components of plant responses to adverse environmental conditions, including host-microbial interactions (142, 144). While host sRNAs play important roles in pathogen resistance, pathogens also encode sRNAs to manipulate host defence responses and mediate virulence (143, 145, 146). The necrotrophic fungus *Botrytis* cinerea infects almost all vegetable and fruit crops, causing major losses worldwide. Recently, it has been reported that some *B. cinerea* sRNAs (Bc-sRNAs) can silence *Arabidopsis* and tomato genes involved in immunity (145). The produced Bc-sRNAs hijack the host RNA interference (RNAi) machinery by binding to *Arabidopsis* AGO1. Furthermore, Bc-sRNAs silence host target immunity genes in both *Arabidopsis* and tomato plants during fungal infection (145). Cross-kingdom RNAi to suppress host immunity genes by hijacking host AGO1 has also been reported for *V. dahliae* (143). *Arabidopsis* ago1-27 mutants were less susceptible to the infection with *V. dahliae* than wild-type plants in both soil and root culture conditions (143). These results indicate that fungal pathogens and hosts utilize cross-kingdom RNAi to manipulate their interactions to their own benefit.

Evolution of pathogen virulence

As effectors are pathogen molecules that are crucial for establishing the parasitic symbiosis, hosts continuously evolve to intercept pathogen effectors or their activities with their immune receptor repertoire to halt pathogen ingress (9). To avoid or overcome such recognition, pathogens need to be able to swiftly purge or modify effectors that are intercepted by host immune systems, or evolve novel effectors to suppress the reinstated immune response, leading to an everlasting co-evolution between pathogen and host (7, 9). Based on genomics of plant pathogenic species, it has been proposed that many pathogens possess a bipartite genome architecture where effector genes cluster in repeat-rich dynamic compartments, a phenomenon that has been coined a "two-speed" genome (147, 148). These regions are typically repeat-rich, sometimes with active transposable elements (TEs), and often display increased structural polymorphism, increased point mutagenesis and positive selection (149-154). TEs are likely to contribute to pathogen adaptation by facilitating the swift evolution of effector catalogs by establishing genetic variability (152, 155), yet the underlying mechanisms remain largely unknown (156). However, genomic analysis in *V. dahliae* revealed active and passive contributions of TEs, through transposable element activity, and through acting as substrate for homology-based double-strand repair pathways, respectively (152).

To control the spread and activity of TEs, TE-rich genomic regions are often highly condensed in heterochromatin, which is directed by DNA methylation. As effector genes and other virulence-related genes, such as toxin biosynthesis genes, often reside in TE-rich regions, TEs can impact the expression of these genes (157-159). Consequently, specific and differential methylation may be associated with adaptive evolution of two-speed pathogen genomes (156, 160, 161). Thus, TEs drive genome and transcriptome variability that, in turn, impacts pathogen adaptation (156).

Conclusion

While all plant pathogenic fungi come across common plant defence mechanisms during host colonization, they employ different strategies to bypass these. As the on-going co-evolution with their hosts prompts pathogens to appropriately respond to modifications in host immunity in a timely manner, fungi need to continuously adapt their repertoire of virulence strategies to keep their parasitic relationships ongoing. A deep understanding of the molecular mechanisms underlying these virulence strategies and of host-pathogen interactions will result in the identification of precise virulence targets in the host plant. Such knowledge is paramount to improve current crop protection strategies or to design novel measures for disease control.

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Chapter 3

Homologs of *Verticillium dahliae* effector Ave1 contribute to virulence of fungal pathogens of diverse plant hosts

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Abstract

Verticillium dahliae is a soil-borne fungal pathogen with a wide host range including many crops. Tomato immune receptor Ve1 confers resistance to *V. dahliae* race 1 strains that express VdAve1. On plants that do not carry Ve1, Ave1 acts as a virulence factor of *V. dahliae*. Homologs of Ave1 are mostly found in plants and in a handful of fungal plant pathogens including *Fusarium oxysporum (FoAve1), Cercospora beticola (CbAve1),* and *Colletotrichum higginsianum (ChAve1)*. In the bacterium *Xanthomonas axonopodis* the Ave1 homolog XacPNP was previously described as a virulence factor. The homologs FoAve1, CbAve1 are recognized by Ve1 although not as efficient as VdAve1. In this study, we analyzed the role of the individual Ave1 homologs in virulence. We generated targeted gene deletions in *Fusarium oxysporum, Cercospora beticola* and *Colletotrichum higginsianum* and showed that both *FoAve1* and *CbAve1* are virulence factors in contrast to *ChAve1* that does not contribute to the virulence of the pathogen. We subsequently tested whether *Ave1* homologs *FoAve1*, *CbAve1*, *ChAve1* and *XacPNP* act in a similar fashion as *VdAve1* by expressing these genes in a *V. dahliae VdAve1* deletion strain. We concluded that VdAve1 on the one hand and FoAve1, CbAve1, ChAve1 and XacPNP on the other hand act in different manners.

Introduction

Plants are surrounded by microbes including oomycetes, fungi, bacteria and viruses. To prevent colonization by harmful microbes, plants evolved pattern recognition receptors (PRRs) that recognize microbe associated molecular patterns (MAMPs) and activate MAMP-triggered immunity (MTI) (1, 2). MAMPs generally are structural components such as bacterial elongation factor Tu (EF-Tu), flagellin, lipopolysaccharides (LPS), fungal chitin and oomycete β-glucans, which are typically conserved across genera of microbes. Successful pathogens secrete effector proteins that modulate host immunity to overcome PRR-mediated plant defence (3, 4). Such effectors are often lineage-specific, facilitate colonization of the plant, and therefore contribute to virulence of the pathogen. In turn, plants evolved immune receptors, typically called resistance (R) proteins, which recognize particular effectors or their activities to mount an immune response known as effector-triggered immunity (ETI) (5). To overcome recognition by the plant, pathogens have to mutate or loose the recognized effector or, alternatively, evolve new effectors to suppress the ETI response. Nevertheless, not all pathogen-secreted molecules follow the strict MAMP-effector dichotomy but rather display traits of either class of molecules, and therefore MTI and ETI responses cannot strictly be separated (6). Typical examples of such pathogen-secreted molecules are Nep1-like proteins, which are virulence factors that are dispersed throughout three kingdoms of life that act as a MAMP at least in Arabidopsis (7, 8). Although less widely distributed, phenomena have been observed for homologs of the Avr4 effector from the tomato leaf mold fungus *Cladosporium fulvum* (9), and for homologs of the Ave1 effector from the vascular wilt fungus *Verticillium dahliae* (4). This has inspired the proposal of the so-called invasion model, which states that plants evolved receptors for all types of molecules, pathogen- as well as host-derived, that can betray microbial invasion to mount the appropriate immune responses (10).

V. dahliae is a soil-borne fungal pathogen that causes vascular wilt in over 200 plant species, including important crop species (11). Resistance to *V. dahliae* is mediated by the immune receptor Ve1 (12, 13), of which homologs were found in mint, hop and cotton amongst other plant species (14-16). Recently, the effector protein that is secreted by *V. dahliae* race 1 strains and that activates Ve1-mediated resistance was identified as Ave1, and all resistance-breaking race 2 strains analyzed thus far lack the complete *Ave1* gene (4, 17). Importantly, *Ave1* deletion strains show reduced aggressiveness on tomato plants that lack Ve1, revealing that *Ave1* acts as a virulence factor on this host species. Additionally, Ave1 was also shown to act as a virulence factor of *V. dahliae* on Arabidopsis plants (4). However, how Ave1 contributes to *V. dahliae* virulence on these plant hosts remains unknown thus far.

Intriguingly, although pathogen effectors are typically lineage-specific traits, many homologs of Ave1 can be found in public databases (4). Remarkably, a wealth of *Ave1* homologs was found in plants as well as in a handful of fungal plant pathogens, including *Fusarium oxysporum (FoAve1), Cercospora beticola (CbAve1) and Colletotrichum higginsianum (ChAve1),* and in the bacterial plant pathogen *Xanthomonas axonopodis (XacPNP)* (4, 18). Interestingly, it was shown that Ve1 is not confined to *V. dahliae* Ave1 (VdAve1), as Ve1 is able to also recognize FoAve1 and CbAve1, while ChAve1 is not recognized (4). Consequently, Ve1 was indeed found to be able to recognize

F. oxysporum in tomato and mount a defense response (4). Remarkably, Ve1 was furthermore found to recognize an endogenous Ave1 homolog (SlAve1) that shares a high degree of identity with VdAve1 (4).

Most plant homologs of Ave1 have been annotated either as plant natriuretic peptides (PNPs) or as expansin-like proteins, and functionally analyzed members were implicated in the regulation of water and ion homeostasis, and consequently in many downstream processes including growth, net water uptake, photosynthesis, stomatal opening and gas exchange (19-22). The observation that the microbial-derived Ave1 homologs do not follow the phylogeny of the species in which they occur is generally taken as evidence for horizontal acquisition, and it has thus been speculated that the microbial Ave1 homologs were acquired from plants (4, 18). In this respect it is interesting that the *X. axonopodis* homolog XacPNP affects homeostasis and photosynthesis in citrus plants, and thus promotes bacterial proliferation as a virulence factor in citrus plants (18, 23, 24). In this study, we investigate whether the Ave1 homologs of several fungal pathogens act as genuine virulence factors.

Results

Characterization of Ve1-mediated tomato defence against Fusarium oxysporum

We previously demonstrated that tomato Ve1 can recognize FoAve1, leading to a defense response that affects infection of *Ve1*-carrying tomato by *F. oxysporum* (4). Because recognition of FoAve1 by *Ve1*-carrying tomato plants does not lead to full immunity against *F. oxysporum* we investigated the occurrence of expression of *FoAve1* in two *F. oxysporum* strains, Bt.01 and Fol4287, in tomato plants lacking *Ve1* with reverse-transcription PCR at 14 DPI. Clear expression of *FoAve1* was monitored at 14 DPI (Figure 1A).

To provide further evidence for the role of FoAve1-recognition in Ve1-mediated tomato defense against Fusarium wilt, we generated *FoAve1* deletion mutants in *F. oxysporum* strains Bt.01 and Fol4287 through homologous recombination. Deletion of *FoAve1* was confirmed by PCR (Suppl. Figure 1A) and the deletion strains were subsequently evaluated for their ability to colonize *Ve1* tomato plants. As anticipated, inoculation of *Ve1* tomato plants with *FoAve1* deletion strains resulted in more extensive symptoms of Fusarium wilt disease when compared to tomato plants inoculated with the corresponding wild type strains (Figure 1B and 1C). Altogether, these data show that tomato Ve1 is able to recognize FoAve1 and activate a defense response that inhibits colonization by *F. oxysporum*, albeit that the response does not fully arrest the fungus and immunity is not fully established. At later stages after inoculation clear symptoms of wilt disease are observed on *Ve1* plants.

FoAve1 is a virulence factor of *F. oxysporum* during tomato colonization

According to the paradigm that plant immune receptors recognize crucial virulence factors of microbial pathogens, it is expected that *FoAve1* contributes to *F. oxysporum* virulence. To test this hypothesis, *FoAve1* deletion strains were inoculated on tomato plants lacking *Ve1*. Indeed,

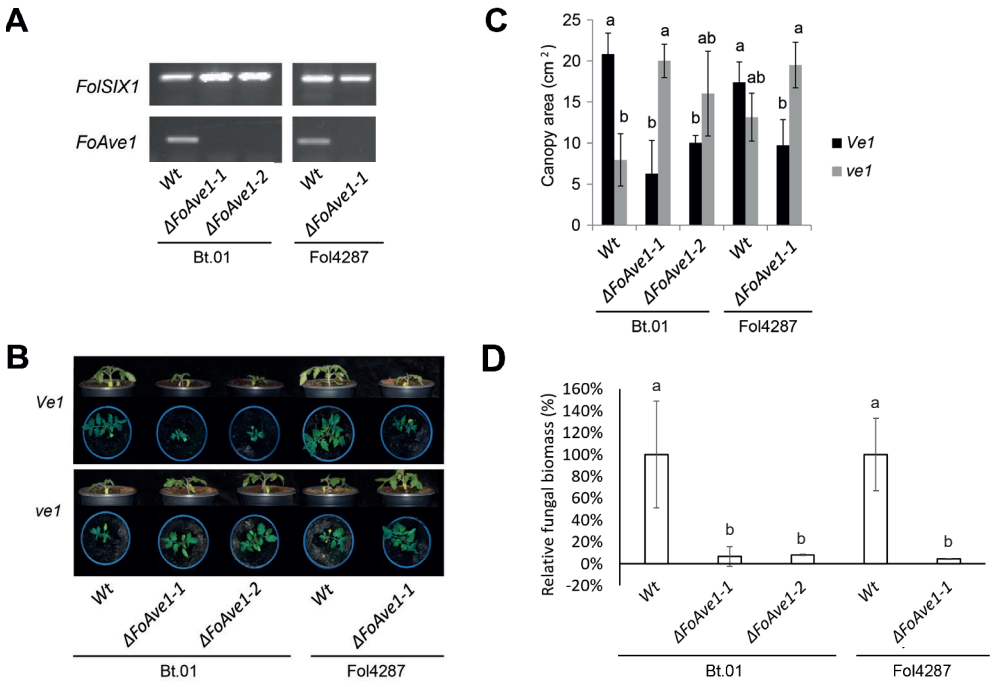

Figure 1. FoAve1 is a virulence factor recognized by tomato Ve1. (A) Expression of FoAve1 in *F. oxysporum* in wild type (Wt) strains Bt.01 and Fol4287 and the FoAve1 deletion strains (Δ*FoAve1*) inoculated on tomato at 14 DPI. (B) (Upper) *FoAve1* deletion strains (Δ*FoAve1*) of *F. oxysporum* strains Bt.01 and Fol4287 escape recognition by tomato Ve1 compared with the corresponding wild type (Wt) strains evidenced by stunted *Ve1* plants at 14 DPI. (Lower) *FoAve1* deletion strains display reduced virulence compared with the corresponding *F. oxysporum* wild type strains Bt.01 and Fol4287 on tomato lacking *Ve1* (*ve1*) evidenced by reduced stunting at 14 DPI. (C) Canopy area of *Ve1* tomato and tomato lacking *Ve1* after inoculation with *FoAve1* deletion strains and the corresponding *F. oxysporum* wild type strains at 14 DPI. (D) *FoAve1* deletion strains display reduced virulence compared to the corresponding *F. oxysporum* wild type strains Bt.01 and Fol4287 on tomato lacking *Ve1* evidenced by reduced fungal biomass at 14 DPI. 8 plants were pooled per 2 plants (n=4). Different letter labels indicate statistically significant differences (P<0.05). Similar results were observed in at least 3 biological replications for all experiments.

FoAve1 deletion strains displayed reduced virulence on tomato plants when compared to the corresponding *F. oxysporum* wild type strain as visualized by the difference in canopy area of the inoculated tomato plants (Figure 1B and 1C). In addition, plants inoculated with *FoAve1* deletion strains resulted in less fungal colonization compared to those inoculated with wild type *F. oxysporum* (Figure 1D). These results suggest that *FoAve1* is a virulence factor of *F. oxysporum*, albeit that its contribution to virulence appears to be relatively minor.

ChAve1 is not a virulence factor of *C. higginsianum* during Arabidopsis colonization

Besides the vascular wilt fungi *V. dahliae* and *F. oxysporum*, that belong to the class of Sordariomycetes, an Ave1 homolog is found in the foliar Sordariomycete *C. higginsianum* (*ChAve1*). Using real-time PCR, *ChAve1* expression was monitored in wild type C. higginsianum during colonization of Arabidopsis plants from 1 to 4 DPI, by which time the plant tissue was completely macerated. No expression of *ChAve1* was detected at any of these time points. Nevertheless, we investigated whether *ChAve1* contributes to virulence in this foliar pathogen. For this purpose, *ChAve1* deletion strains were generated that, after confirmation with PCR (Suppl. Figure 1B), were evaluated for aggressiveness on *Arabidopis thaliana*. As expected *ChAve1* deletion strains did not show reduced virulence when compared to the corresponding wild type *C. higginsianum*, as infections resulted in lesions with similar sizes (Figure 2A). Moreover, similar levels of pathogen biomass were recorded in plants inoculated with the wild-type fungus and the deletion strains (Figure 2B). Together these results show that ChAve1 is not a virulence factor of *C. higginsianum* on Arabidopsis, likely due to the lack of *ChAve1* expression.

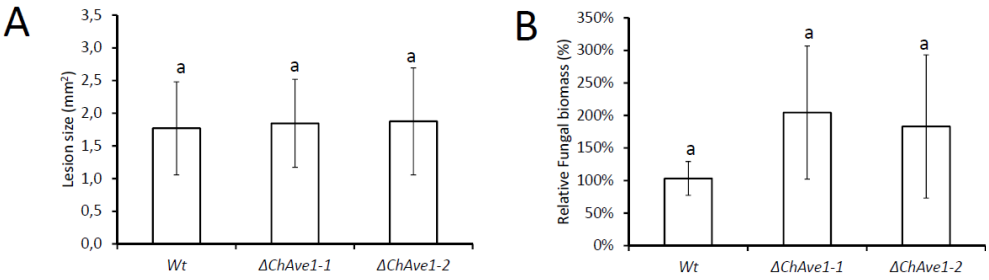

Figure 2. ChAve1 is not a virulence factor of *C. higginsianum*. (A) ChAve1 deletion strains (ΔChAve1) show a similar lesion size compared to the corresponding C. higginsianum wild type strain (Wt) at 3 DPI on 3-week-old A. thaliana. Twenty four lesions on three A. thaliana plants were measured per strain. No significant difference was observed ($P<0.05$). (B) No difference in fungal biomass was observed at 4 DPI on spray inoculated 3-week-old Arabidopsis plants (n=4). Different letter labels indicate statistically significant differences ($P<0.05$). Similar results were observed in at least 3 biological replications for all experiments in this figure.

CbAve1 is a virulence factor of *C. beticola* during sugar beet colonization

Besides Sordariomycetes, Ave1 homologs were also identified as we previously identified an Ave1 homolog in the Dothidiomycete *C. beticola* (*CbAve1*). Expression of *CbAve1* was investigated in *C. beticola* during colonization of sugar beet plants between 3 and 18 DPI using real-time PCR. Clear expression was detected between these time points. To evaluate the contribution

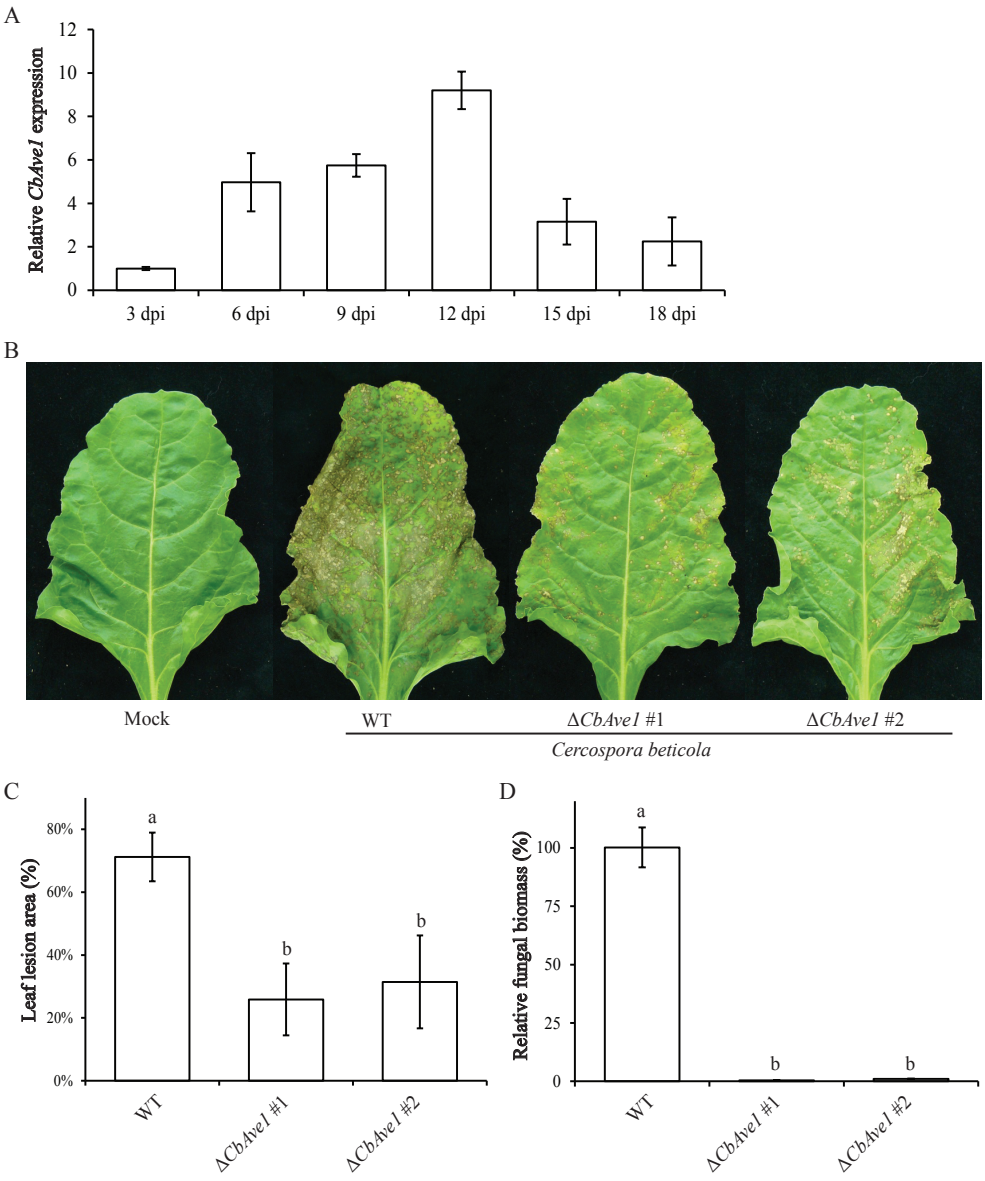

Figure 3. CbAve1 is a virulence factor of *C. beticola*. (A) Expression of *CbAve1* during infection of *C. beticola* on sugar beet. Leaves of 6- to 7-week-old sugar beet plants were inoculated with wild-type *C. beticola* and collected at regular intervals from 3 to 18 days post inoculation (DPI). RT-qPCR was performed to determine the relative expression levels of *CbAve1* using the *C. beticola actin* gene as a reference, and compared with *CbAve1* expression in *C. beticola* in sugar beet plants upon the wild-type *C. beticola* inoculation at 3 DPI, which is set to 1. (B) *CbAve1* deletion strains (Δ*CbAve1* #1 and Δ*CbAve1* #2) show reduced virulence compared to the corresponding *C. beticola* wild type strain (WT) visualized by a reduction in lesion area on sugar beet leaves at 15 DPI. (C) Quantification of leaf lesion caused by *C. beticola* on sugar beet plants at 15 DPI (n>5). Bars represent the average percentage of leaf lesion area of whole leaf area with standard deviations. (D) Fungal biomass determined with qPCR in *Cercospora*-inoculated sugar beet plants at 15 DPI. The fungal biomass in sugar beet plants upon inoculation with the wild-type *C. beticola* is set to 100 % (control). Different letter labels indicate statistically significant differences (P<0.05).

of *CbAve1* in *C. beticola* virulence, *CbAve1* deletion strains were generated and confirmed with PCR. For this pathogen, deletion of the *Ave1* homolog resulted in reduced virulence compared to the corresponding wild type strain, as infection resulted in reduced numbers and size of the lesions (Figure 3A; B). As expected, *CbAve1* deletion strains showed reduced colonization on sugar beet plants compared to the corresponding *C. beticola* wild type strain (Figure 3C). Taken together, these results show that CbAve1 is a virulence factor of *C. beticola*.

Functional diversification among plant and fungal Ave1 homologs

VdAve1 is a virulence factor of *V. dahliae* that shows a high degree of identity with homologs from plants. It has therefore been proposed that *VdAve1* has been acquired by *V. dahliae* from plants through horizontal gene transfer (4). However, the function of VdAve1 through which it contributes to *V. dahliae* aggressiveness remains presently enigmatic. Similarly, the function of the Ave1 homologs that are found in various pathogens also remains unclear. In order to evaluate whether all homologs share their functionality, we tested whether they can complement the virulence defect that is observed upon *VdAve1* deletion in *V. dahliae*. To this end, we transformed one of the *VdAve1* deletion strains of *V. dahliae* with constructs to drive expression of the plant homologs derived from tomato (*S. lycopersicum; SlAve1*) and grape (*V. vinifera; VvAve1*) by the *V. dahliae VdAve1* promotor (4). To confirm that the plant homologs were expressed in *V. dahliae* upon colonization of tomato we monitored *SlAve1* and *VvAve1* expression with PCR. At 14 DPI we monitored clear expression of *SlAve1* and *VvAve1* in 3 transformants for each construct (Suppl. Figure 2a). Subsequently, we evaluated the ability of these transformants to cause disease on tomato plants lacking *Ve1*. All *VdAve1* deletion strains carrying *SlAve1* and *VvAve1* showed a similar phenotype and fungal colonization as the corresponding *VdAve1* deletion strain (Figure 4; Suppl. Figure 2). These results suggest that two plant Ave1 homologs that share a high identity with VdAve1 are unable to restore virulence in a *VdAve1* deletion strain, suggesting that Ave1 homologs from plants act in a different manner as VdAve1.

Subsequently, we evaluated whether microbial Ave1 homologs can reinstall the compromised virulence of *V. dahliae* that results from *VdAve1* deletion. To this end, we transformed one of the *VdAve1* deletion strains with constructs to drive expression of the Ave1 homologs derived from *F. oxysporum* (*FoAve1*), *C. higginsianum* (*ChAve1*) and *C. beticola* (*CbAve1*) by the VdAve1 promoter. We also included the Ave1 homolog derived from the plant pathogenic bacterium *Xanthomonas axonopodis* (*XacPNP*) that was previously described as a virulence factor (18). We confirmed in a minimum of 2 transformants that the constructs were expressed (Suppl. Figure 2a). Subsequently, we tested the *V. dahliae* transformants on tomato lacking *Ve1* and compared them with the corresponding wild type *V. dahliae* and the *V. dahliae VdAve1* deletion strain complemented with *VdAve1*. *VdAve1* deletion strains expressing *FoAve1, CbAve1, ChAve1 and XacPNP* showed a similar disease phenotype and fungal colonization as *VdAve1* deletion strains (Figure 4; Suppl. Figure 2). (Figure 4; Suppl. Figure 2). These results show that the *Ave1* homologs *FoAve1, CbAve1, ChAve1 and XacPNP* cannot restore virulence in a *VdAve1* deletion strain which may suggest that Ave1 homologs of *V. dahliae* on the one hand, and *F. oxysporum, C. beticola, C. higginsianum and X. axonopodis* on the other hand, contribute to fungal virulence in a different manner.

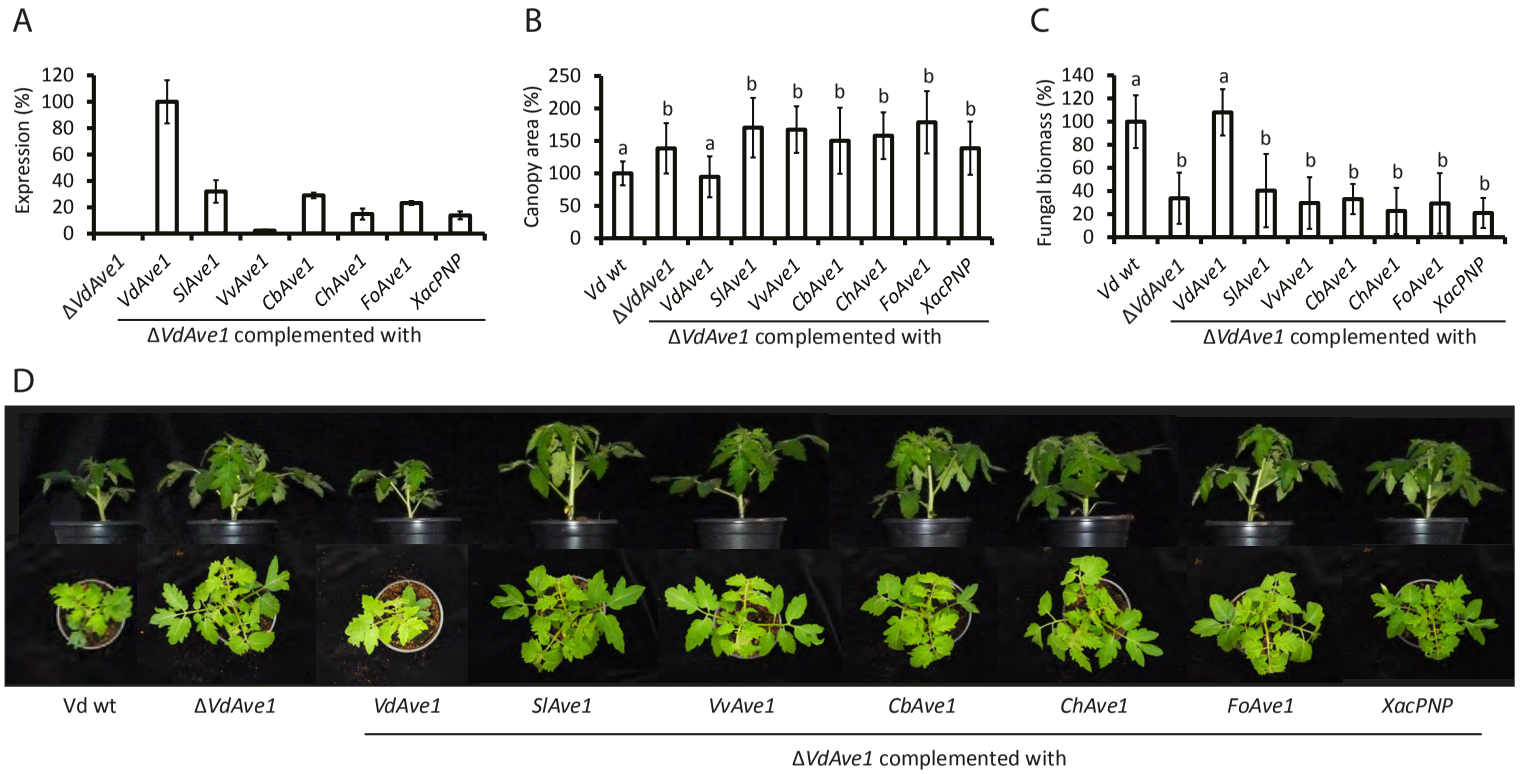

Figure 4. VdAve1 acts in a different manner than Ave1 homologs derived from plant pathogens. *V. dahliae VdAve1* deletion strain (Δ*VdAve1*) complemented with *Ave1* homologs derived from *V. dahliae* (*VdAve1*), tomato (*S. lycopersicum*; *SlAve1*), grape (*V. vinifera*; *VvAve1*), *F. oxysporum* (*FoAve1*), *C. beticola* (*CbAve1*), *C. higginsianum* (*ChAve1*) and *Xanthomonas axonopodis* (*XacPNP*) inoculated on tomato plants. (A) Gene expression of Ave1 homologs by V. dahliae during colonization of tomato at 14 dpi. (B) Canopy area of tomato plants inoculated with *V. dahliae* strains expressing *Ave1* homologs compared to *V. dahliae* wild type (Vd wt) at 14 dpi. (C) Fungal colonization of tomato plants inoculated with *V. dahliae* strains expressing *Ave1* homologs compared to *V. dahliae* wild type (Vd wt) at 14 dpi. Different letter labels indicate significant differences ($P<0.05$). (D) Photos taken from the side and the top of tomato plants inoculated with *V. dahliae* strains expressing *Ave1* homologs compared to *V. dahliae* wild type (Vd wt) at 14 dpi.

Discussion

The role of FoAve1 in recognition by tomato immune receptor Ve1

We have previously shown that recognition of FoAve1 by Ve1 leads to a defense response (4) and that Ve1 tomato plants can recognize *F. oxysporum*. To confirm that FoAve1 is involved in recognition we first analyzed *FoAve1* expression. A previous study could not detect *FoAve1* expression in *F. oxysporum* after inoculation on tomato (25). However, in the conditions that we tested clear *FoAve1* expression was monitored in *F. oxysporum* on tomato at 14 DPI. In addition, deletion of *FoAve1* resulted in abolishment of recognition by Ve1. Our data suggest that the incomplete disease resistance cannot be attributed to a lack of *FoAve1* expression, but may be the result of a less efficient detection of this Ave1 homolog by Ve1. Similar differences in recognition efficiencies were previously observed for *Cladosporium fulvum* effector Ecp2 and its homolog MfEcp2 of *Mycosphaerella fijiensis* when coexpressed with tomato immune receptor Cf-Ecp2 (9).

Some Ave1 homologs act as virulence factors

Previously, we have shown that Ave1 is a virulence factor of *V. dahliae* (4). In this study, we tested wheter Ave1 homologs in other plant pathogens act as virulence factors as well. We showed that deletion of *Ave1* homologs in *F. oxysporum* and *C. beticola* resulted in reduced aggressiveness on their respective plant hosts. In contrast, the Ave1 homolog *ChAve1* of the foliar pathogen *C. higginsianum* does not contribute to virulence due to the lack of expression in *planta*. Possibly, the lack of expression is due to the availability of effectors with a similar function that render ChAve1 functionality redundant. Alternatively, recognition of ChAve1 by a host immune receptor posed pressure on *C. higginsianum* to avoid recognition, resulting in the lack of *ChAve1* expression. Interestingly, homologs of tomato Ve1 have been identified in many other plant species, of which some within as well as outside the Solanaceae family have been shown to be functional immune receptors (26).

Functional diversification among Ave1 homologs

Since Ave1 homologs derived from plants display high similarity to Ave1, it has been suggested that Ave1 was acquired through horizontal gene transfer from plants (4). As the function of the Ave1 homologs remain unknown we tested whether the Ave1 homologs share the same functionality by complementing a *V. dahiae Ave1* deletion strain with homologs derived from plants and various plant pathogens. Highly similar Ave1 homologs derived from plants are unable to restore virulence in an Ave1 deletion strain. Therefore we conclude that Ave1 homologs derived from plants act in a different manner as Ave1. Similarly, Ave1 homologs derived from *F. oxysporum, C. beticola, C. higginsianum and X. axonopodis* cannot restore the virulence penalty caused by the deletion of Ave1. Therefore, we conclude that Ave1 from *V. dahliae* on the one hand, and Ave1 homologs derived from *F. oxysporum, C. beticola, C. higginsianum and X. axonopodis* on the other hand, contribute to virulence in a different manner. Similarly, functional diversification within effector families has been observed for LysM and NLP effectors (27, 28). Alternatively, it is important to note that *V. dahliae, C. beticola, C. higginsianum and X. axonopodis* have diverse plant hosts and that the reason for the inability to restore virulence in an *V. dahliae Ave1* deletion strain could be that each homolog targets a host-specific protein. This would for example explain why the homolog of *C. beticola* that likely

targets a sugar beet protein cannot restore virulence in an *V. dahliae Ave1* deletion strain colonizing tomato. Host specific effector adaptation has been suggested for the closely related oomycetes *Phytophtora infestans and P. mirabilis* that are pathogens on different hosts. Homologous effectors of both pathogens act more efficiently on their specific host target than on a "foreign" host target (29). Nevertheless, the finding that the *Ave1* homolog from *F. oxysporum* f.sp. *lycopersici* cannot complement the *VdAve1* deletion strain for virulence on tomato argues against this hypothesis.

Materials and Methods

Deletion strains

Deletion strains were generated in *F. oxysporum* and *C. higginsianum* by amplifying 1,5 kb sequences (Suppl. Table 1) flanking the coding sequence of *FoAve1* and *ChAve1*, respectively, and cloned as previously described (30) into vector pRF-HU2 containing a nourseothricin cassette for selection. For the complementation of *V. dahliae Ave1* deletion strains *FoAve1, CbAve1, ChAve1, SlPNP, VvPNP and XacPNP* were obtained by gene synthesis (Eurofins Genomics, Ebersberg, Germany). The synthesized genes were then cloned into vector pFBT005 under the VdAve1 promoter, containing a nourseothricin cassette for selection. *F. oxysporum* (Fol4287 and Bt.01) and *C. higginsianum* (IMI349063A) conidiospores were transformed on a Hybond-N+ filter by *A. tumefaciens* carrying the pRF-HU2 plasmid containing the constructs and a *V. dahliae Ave1* deletion strain (JR2) was transformed by *A. tumefaciens* carrying the pFBT005 plasmid containing the constructs as previously described (31). Transformants were then selected on potato dextrose agar (PDA) (Thermo Fisher Scientific Inc, Breda, The Netherlands) containing hygromycin B (Duchefa Biochemie BV, Haarlem, The Netherlands) for selection of *F. oxysporum, C. beticola and C. higginsianum transformants* or nourseothricin sulphate (Sigma Aldrich Chemie BV, Zwijndrecht, The Netherlands) for selection of *V. dahliae* transformants. After five to seven days at room temperature, individual transformants were transferred from the filter to fresh PDA plates with the appropriate selection and incubated for ten days (31). To verify the transformants, fungal spores were collected and genomic DNA was extracted followed by a PCR to test the presence of the hygromycin or nourseothricin cassette and the presence of the construct in the genome (Suppl. Table 1).

CbAve1 deletion mutants of *C. beticola* were generated using the split-marker approach described by Catlett, et al. (32). Genomic DNA of the wild type *C. beticola* strain 09-40 and pDAN vector (33) served as PCR templates to generate split-marker PCR constructs used for transformation. Primers are listed in Suppl. Table 1. PEG-mediated transformation of the wild type *C. beticola* strain 09-40 was performed as previously described (34). Site-directed gene replacement was confirmed by the absence of PCR product using split-marker CbAve1 1F forward primer of the 5' flank of the target gene and MDB-760 reverse primer designed on the coding sequence of *CbAve1*.

Pathogen inoculations

Plants were grown in soil in the greenhouse at 21oC/19oC during 16-h/8-h day/night periods, respectively, with 70% relative humidity and 100 W m-2 supplemental light when the intensity dropped below 150 W m-2.

For *V. dahliae* and *F. oxysporum* inoculations, 10-day-old tomato (*Solanum lycopersicum*; cv. MoneyMaker or MoneyMaker *35S:Ve1*) (13) were uprooted, rinsed in water and dipped for 5 minutes in a suspension of 106 conidiospores per mL of water harvested from 1- to 2-week-old *V. dahliae* or *F. oxysporum* cultures on PDA as previously described (13). Control plants received the same treatment, but the roots were dipped in water without conidiospores. After replanting in fresh soil, plants were incubated at standard greenhouse conditions. Disease development was monitored up to 21 days post inoculation (DPI).

For *C. higginsianum* inoculations 3-week-old Arabidopsis (*A. thaliana* ecotype Co-0 or Col-0 35S:Ve1) (35) were inoculated on the leaves with either 2 µl drops or sprayed with a suspension of 106 conidia per mL of water harvested from 1- to 2-week-old *C. higginsianum* cultures on Mathurs' agar as previously described (36, 37). Control plants received the same treatment, but the leaves were sprayed with water without conidiospores. After sealing the plants inside a transparent closed box lined with wet tissue paper to provide high humidity, they were incubated at 25°C, under a 16-h/ 8-h light/dark regime (36). Disease development was monitored up to 4 DPI.

For *C. beticola* inoculations 6- to 7-week-old sugar beet plants (*Beta vulgaris*) were inoculated by evenly spaying spore suspension of 105 conidia per mL of water on the lower side of the leaves harvested from *C. beticola* cultures growing on V8 solid medium as previously described (38). Control plants received the same treatment, but the leaves were sprayed with water. Plants were incubated at standard greenhouse conditions and disease development was monitored up to 18 DPI.

Fungal biomass and gene expression

Stem sections of tomato, cut from the base of the stem up to the cotyledons, were collected at 7 and 14 DPI from plant inoculated with *F. oxysporum* or *V. dahliae*. Arabidopsis leaves were collected at 1-4 DPI after inoculation with *C. higginsianum*. Sugar beet leaves were collected at regular intervals between 3 and 18 DPI from plants inoculated with *C. beticola*. Collected plant tissue was flash frozen in liquid nitrogen and ground to powder, of which an aliquot of ~100 mg was used for RNA extraction with the Quick-RNATM Miniprep kit (Zymo Research Europe GmbH, Freiburg, Germany), and cDNA was synthesized using M-MLV Reverse Transcriptase (Promega Benelux BV, Leiden, The Netherlands). Fungal biomass was determined by quantifying the expression of *VdGAPDH, FoTUB, ChTUB and CbAct* relative to the expression of *SlRUB* (tomato), *AtRub* (Arabidopsis), *BvAct* (sugar beet), respectively. Expression of *VdAve1, FoAve1, CbAve1, ChAve1, SlPNP and VvPNP* was determined by quantifying the expression relative to the expression of *VdGAPDH, FoTUB, ChTUB and CbAct*, respectively (Suppl. Table 1). Real-time PCR was carried out on an ABI7300 PCR machine (Applied Biosystems, Life Technologies Europe BV, Bleiswijk, The Netherlands) in combination with the qPCR SensiMix kit (BioLine, GC Biotech BV, Alphen aan den Rijn, The Netherlands). The following Real-time PCR conditions were used: an initial 95°C denaturation step for 10 minutes followed by denaturation for 15 seconds at 95°C, annealing for 60 seconds at 60°C, and extension at 72°C for 40 cycles.

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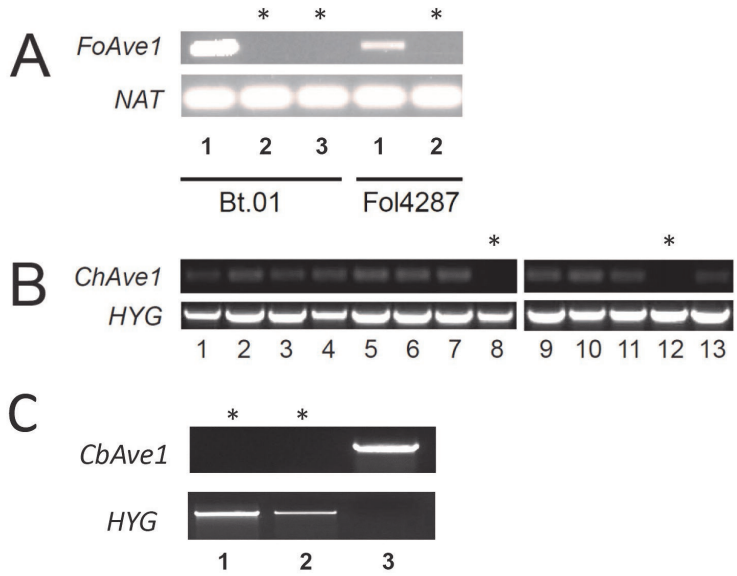
Supplementary data

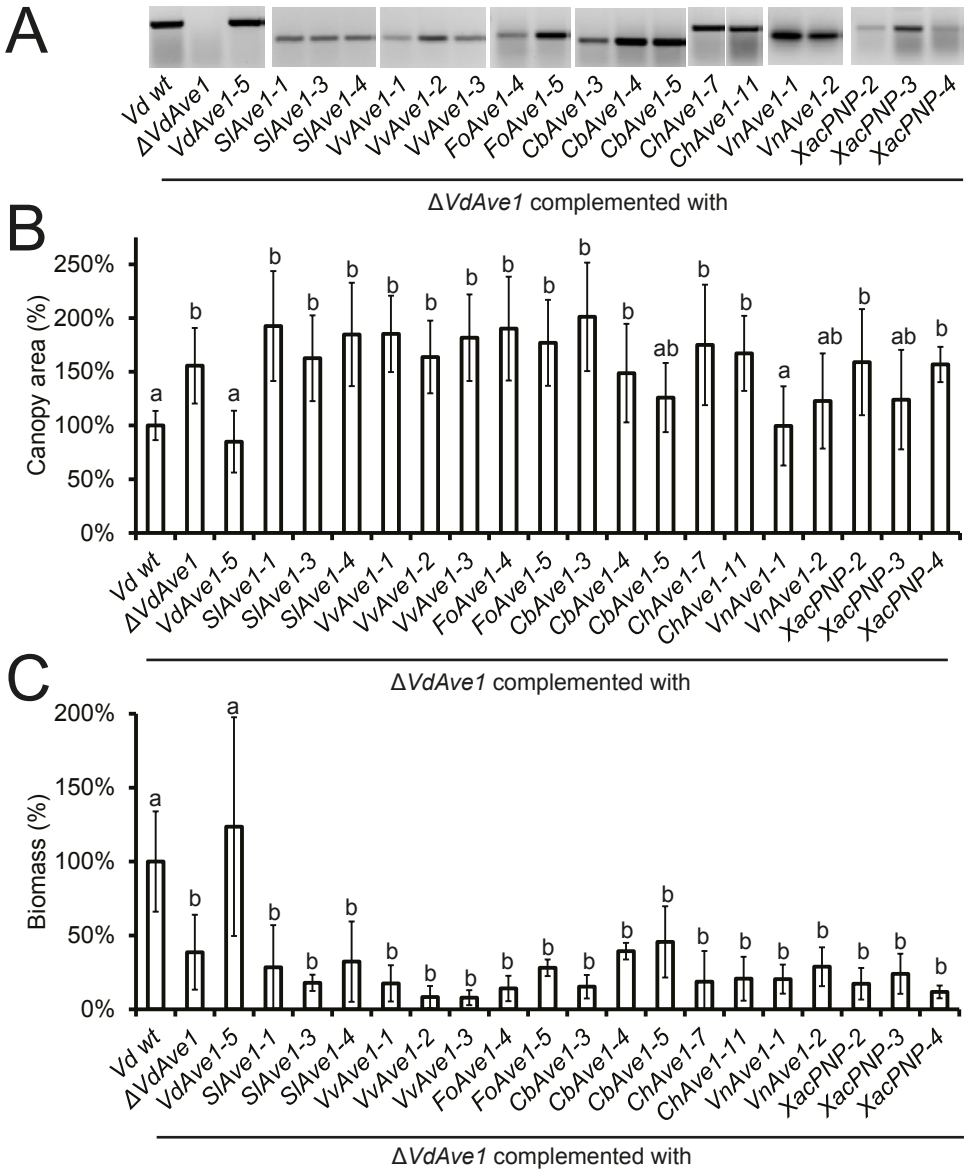
Suppl. Table 1. Primers used in this study

Primer	Sequence (5'- 3')	Notes
SlRub_QPCR_F	GAACAGTTTCTCACTGTTGAC	*S. lycopersicum Rubisco*
SlRub_QPCR_R	CGTGAGAACCATAAGTCACC	*S. lycopersicum Rubisco*
FolSIX1_F	GTCTCACGAGCCAAGTCTACC	*F. oxysporum Six1*
FolSIX1_R	GAACCGCAGCCTCTTGAGCAT	*F. oxysporum Six1*
FolTub_F	CTCTGGCAACAAGTATGTTCCC	*F. oxysporum Tubulin*
FolTub_R	TTGTCGGGACGGAAGAGCTGA	*F. oxysporum Tubulin*
FolAve1_QPCR_F3	ATATCGGAACTGCAAATATTCTCAAC	*F. oxysporum Ave1*
FolAve1_QPCR_R3	CTTATACATTTCATCGTATACAGTCTGC	*F. oxysporum Ave1*
AtRub_QPCR_F	GCAAGTGTTGGGTTCAAAGCTGGTG	*A. thaliana Rubisco*
AtRub_QPCR_R	CCAGGTTGAGGAGTTACTCGGAATGCTG	*A. thaliana Rubisco*
ChELF1a_F	CTGGTACAAGGGTTGGGAGA	*C. higginsianum Elongation factor*
ChELF1a_R	ACCGCCGATCTTGTAGACAT	*C. higginsianum Elongation factor*
ChAve1_QPCR_F5	CAAGATGCTATGGCAACAATATGAAC	*C. higginsianum Ave1*
ChAve1_QPCR_R5	GTCTTGAGGAAAATCTATCGTATTTCTG	*C. higginsianum Ave1*
BvAct_QPCR_F	GATTTGGCACCACACCTTCT	*B. vulgaris actin*
BvAct_QPCR_R	TCTTTTCCCTGTTTGCCTTG	*B. vulgaris actin*
CbAct_QPCR_F	ACATGGCTGGTCGTGATTTG	*C. beticola actin*
CbAct_QPCR_F	TGTCCGTCAGGAAGCTCGTA	*C. beticola actin*
CbAve1_QPCR_F	ATTCCCTTCAGGCAACCTCT	*C. beticola Ave1*
CbAve1_QPCR_R	CGGACAAGCTTCGCAATAAT	*C. beticola Ave1*
VdGapdh_F	CGAGTCCACTGGTGTCTTCA	*V. dahliae GAPDH*
VdGapdh_R	CCCTCAACGATGGTGAACTT	*V. dahliae GAPDH*
VdAve1-Fw5	ATCCTACTATAACCCACCCTACCTTC	*V. dahliae Ave1*
VdAve1-Rv5	CATCATATGAGTCCTGAGATAAGATCA	*V. dahliae Ave1*
XacAve1_QPCR_F	GCAATCGGTTTGCTCTTTTC	*X. axonopodis XacPNP*
XacAve1_QPCR_R	AGCACCGTTATCCCACAGAC	*X. axonopodis XacPNP*
VvAve1-QPCR-F2	CGTTATAGGTTAAGGTGCCTGAGT	*V. vinifera Ave1*
VvAve1-QPCR-R2	TTCATGCTAGGAGAGTGTGAAATG	*V. vinifera Ave1*
SlAve1_QPCR_F	CGTCGGGGAATCTATTTGTG	*S. lycopersicum Ave1*
SlAve1_QPCR_R	AAAGCATCCGTTGACAAAGC	*S. lycopersicum Ave1*
FolAve1_LB_F3	GGTCTTAAUAACCTAACCTGTATCTAGACCAGAGTGTCT	Left border *FolAve1*
FolAve1_LB_R3	GGCATTAAUCAGCAGGTGTTTAGGTATTGTTAAGATAAG	Left border *FolAve1*
FolAve1_RB_F	GGACTTAAUGTTCTTAGAGCTTAAGGTCAATGTAGC	Right border *FolAve1*
FolAve1_RB_R	GGGTTTAAUCTGCAGCTTTCACGGGGCTA	Right border *FolAve1*
ChAve1_LB_F	GGTCTTAAUGGCAGGAGGTCTAGGTGAGA	Left border *ChAve1*
ChAve1_LB_R	GGCATTAAUCGACTTGAGTTTTGCTGCAC	Left border *ChAve1*
ChAve1_RB_F	GGACTTAAUAAAGATTTTCGCAGTGCTTCA	Right border *ChAve1*

ChAve1_RB_R	GGGTTTAAUAGGCTGCAGAATGAGTTTCG	Right border *ChAve1*
pRF-HU2_Hyg_Fw	CTATTCCTTTGCCCTCGGACGAGTGC	Hygromycin primers
pRF-HU2_Hyg_Rv	CGATGTAGGAGGGCGTGGATATGTCC	Hygromycin primers
NAT_Fw	CGGGCCGGATTGGTCAAGATTTGC	Nourseothricin primers
NAT_Rv	CGATTCGTCGTCCGATTCGTCG	Nourseothricin primers
MDB-277	GACGTTGTAAAACGACGGCCAGTG	Split-marker HYG-F
MDB-258	GGATGCCTCCGCTCGAAGTA	Split-marker HY-R
MDB-259	CGTTGCAAGACCTGCCTGAA	Split-marker YG-F
MDB-278	CACAGGAAACAGCTATGACCATGA	Split-marker HYG-R
MDB-1145	GGCAGGTAGATGACGACCAT	HYG R2
CbAve1 1F	CAAGATTGGGCCTTCGTATG	*CbAve1* split-marker left border
CbAve1 2R	CACTGGCCGTCGTTTTACAACGTCTCTTCAATGGATCCGGACTG	*CbAve1* split-marker left border
CbAve1 3F	TCATGGTCATAGCTGTTTCCTGTGTGAGTGGGGTTTTTGGTTTC	*CbAve1* split-marker right border
CbAve1 4R	AAGCATACCTCTTCGGCAAA	*CbAve1* split-marker right border
MDB-760	CAACGTCTGCCACAAGCTGCT	*CbAve1* reverse



Suppl. Figure 1. Verification of deletion strains. (A) Identification of *F. oxysporum FoAve1* deletion strains by amplification of *FoAve1* and selection marker nourseothricin (*NAT*) from DNA obtained from transformants. DNA bands were observed at the expected size. Asterisks indicate positive transformants, while transformants that lack an asterisk are ectopic transformants. (B) Identification of *C. higginsianum ChAve1* deletion strains by amplification of *ChAve1* and selection marker hygromycin (*HYG*) from DNA obtained from *C. higginsianum* transformants. DNA bands were observed at the expected size. Asterisks indicate positive transformants, while transformants that lack an asterisk are ectopic transformants. (C) Verification of *CbAve1* deletion strains (1 and 2) based on absence of *CbAve1* using gene-specific primers and presence of the hygromycin resistance cassette. Amplification on *C. beticola* Wt gDNA (3) as a control showing the presence of *CbAve1* and absence of the hygromycin resistance cassette at the expected size. Asterisks indicate positive transformants.



Suppl. Figure 2. VdAve1 acts in a different manner than Ave1 homologs derived from plant pathogens. *V. dahliae VdAve1* deletion strain (Δ*VdAve1*) complemented with *Ave1* homologs derived from *V. dahliae* (*VdAve1*), tomato (*S. lycopersicum; SlAve1*), grape (*V. vinifera; VvAve1*), *F. oxysporum* (*FoAve1*), *C. beticola* (*CbAve1*), *C. higginsianum* (*ChAve1*) and *Xanthomonas axonopodis* (*XacPNP*) inoculated on tomato plants. (A) Expression of *VdAve1*, *SlAve1*, *VvAve1*, *FoAve1*, *CbAve1*, *ChAve1 and XacPNP* in a minimum of 2 transformants. (B) Canopy area of tomato plants inoculated with *V. dahliae* strains expressing *Ave1* homologs in a minimum of 2 transformants compared to *V. dahliae* wild type (Vd wt) at 14 DPI. (C) Fungal colonization of tomato plants inoculated with *V. dahliae* strains expressing *Ave1* homologs compared to *V. dahliae* wild type (Vd wt) at 14 DPI. Different letter labels indicate significant differences (P<0.05). (D) Photos taken from the side and the top of tomato plants inoculated with *V. dahliae* strains expressing *Ave1* homologs compared to *V. dahliae* wild type (Vd wt) at 14 DPI.

Chapter 4

Identification of *Cercospora beticola* necrosis-inducing effector CbNip1

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Ronnie de Jonge, Jonathan D. Neubauer, Gary A. Secor,
Bart P.H.J. Thomma, and Melvin D. Bolton

Abstract

Cercospora beticola is a hemibiotrophic fungus that causes Cercospora leaf spot disease of sugar beet (*Beta vulgaris* L.). After an initial, symptom-free, biotrophic phase of colonization, necrotic lesions appear on the host leaves as the fungus switches to a necrotrophic lifestyle. The phytotoxic secondary metabolite cercosporin has been shown to facilitate fungal virulence for several *Cercospora* spp. However, since cercosporin production and subsequent cercosporin-initiated formation of reactive oxygen species is light-dependent, cell death evocation by this toxin is only fully ensured during a period of light. Here, we report the discovery of the effector protein CbNip1 secreted by *C. beticola* that can cause necrosis in the absence of light and therefore may complement light-depended necrosis formation by cercosporin by inducing necrosis during periods of darkness. Infiltration of CbNip1 protein into sugar beet leaves reveals that darkness is essential for full CbNip1-triggered necrosis, as light exposure delayed CbNip1-triggered host cell death. Gene expression analysis during host infection shows that *CbNip1* expression is correlated with symptom development in *planta*. Targeted gene replacement of *CbNip1* leads to a significant reduction in virulence indicating the importance of CbNip1 during colonization.

Introduction

Cercospora leaf spot (CLS) disease is considered one of the most destructive foliar disease of sugar beet worldwide (1). The causal agent of CLS is the hemibiotrophic fungus *Cercospora beticola* that belongs to the class of Dothideomycetes (2). In the field, *C. beticola* over-winters as stromata (1, 3, 4). As *C. beticola* conidia are airborne, inoculum is dispersed throughout the field by wind, rain and insect transfer (1, 3). Upon landing on a sugar beet leaf, spores germinate and grow towards stomata where they form appressoria (1, 5, 6). These hyphal structures enable the fungus to penetrate and enter the apoplast (7). Once inside the host, *C. beticola* grows intercellularly and colonizes the mesophyll (1). During these early stages of infection, *C. beticola* lives a biotrophic lifestyle (1). However, unknown conditions trigger hemibiotrophic fungi to switch from a biotrophic to a necrotrophic lifestyle in which they induce host cell death to complete their lifecycle (7, 8).

Necrosis-inducing molecules come in many forms and with various modes of actions. For example, necrotrophic effectors, also known as proteinaceous host-selective toxins, depend on the presence of a corresponding target encoded by a susceptibility gene in their host to elicit host cell death (9-12). This interaction is essentially the classic gene-for-gene interaction (13), but instead of providing resistance to the fungus, host cell death serves the necrotrophic needs of the fungus. Therefore, this interaction is also referred to as an inverse gene-for-gene interaction (9). For example, the necrotrophic effector SnTox1 of the wheat pathogen *Parastagonospora nodorum* interacts with Snn1 encoded by a wheat receptor kinase gene, which activates programmed cell death in the host and facilitates a compatible interaction (11, 14, 15). However, not all necrosis-inducing effectors are dependent on a host receptor to provoke host cell death. A family of Nep1-like proteins (NLPs) has been identified in several oomycetes, fungi and bacteria that elicit a hypersensitive response-like host necrosis (16, 17). The first family member discovered was Nep1 (necrosis and ethylene inducing protein 1), a 24 kDa protein secreted by *Fusarium oxysporum* that was shown to trigger necrosis and ethylene production in *Erythroxylum coca* (coca plant) (18). Besides high sequence homology, NLPs share a common NPP1 (necrosis-inducing *Phytophthora* protein) domain (19). Motteram *et al*. (2009) (20) reported a NPP1 domain carrying phytotoxic effector called MgNLP that is expressed during infection of the hemibiotrophic pathogen *Zymoseptoria tritici*, the causal agent of septoria tritici blotch on wheat. Furthermore, necrosis-inducing activity was described as selective since MgNLP induced cell death in *Arabidopsis* and tobacco but not in wheat. Interestingly, targeted gene replacement of MgNLP did not affect fungal virulence in inoculation studies of susceptible wheat lines (20). Additionally, analysis of *Z. tritici* culture filtrates led to the discovery of two light-dependent phytotoxic proteins, ZtNIP1 and ZtNIP2, whose activities resemble those of host-specific toxins (21). While *ZtNIP1* displays homology to the *Cladosporium fulvum* effector protein Ecp2 that is known to elicit cell death in tomato and tobacco harboring the *Cf-ECP2* resistance gene (22), *ZtNIP2* was identified to contain a putative MD-2-related lipid-recognition domain hinting at the ability to bind lipids that may have a potential role in innate immunity (23, 24). Furthermore, the onset of *ZtNIP1* expression during infection matched with necrotic symptom development in *planta* (21). Recently, the functional ribonuclease Zt6 was discovered in *Z. tritici* that targets not only plant but also mammalian ribosomal RNA for cleavage *in vitro*, a feature that makes it highly toxic to wheat,

tobacco, bacterial and yeast cells (25). Intriguingly, the gene expression pattern of *Zt6* during infection is marked by a double expression peak. The first boost in expression occurs at one day post infection, followed by down-regulation during the biotrophic life cycle phase. With onset of the necrotrophic phase at 14 DPI, however, *Zt6* gene expression increases again (25).

Besides proteinaceous necrosis-inducing agents, secondary metabolite (SM) effectors have also been reported to elicit cell death in their host. *C. beticola* is a producer of cercosporin and beticolin, two well-known phytotoxic SMs. Both toxins are only active in the presence of light and show no host specificity (26, 27). In multiple *Cercospora* species, targeted gene disruption mutants that are unable to produce cercosporin displayed reduced virulence which underlines the importance of necrosis induction for the infection process in this genus (28, 29). However, no proteinaceous phytotoxin has been reported for *C. beticola* to our knowledge. In this study, we describe the identification of the first proteinaceous *C. beticola* virulence factor, which is able to induce host cell death in the dark and therefore can complement the light-dependent phytotoxins cercosporin and beticolin.

Results

Necrosis-inducing activity of *C. beticola culture* filtrate

Due to the hemibiotrophic lifestyle of *C. beticola*, we hypothesized that the fungus secretes effector proteins during infection that facilitate disease by causing necrosis and at least a portion of these are produced during *in vitro* growth. Therefore, we cultured *C. beticola* in different media (PDB and Fries medium) and under different conditions (shaking/still cultures and sampling time points at 3, 5, 7, 12, and 14 days after medium inoculation) in attempts to identify an *in vitro* condition in which effector proteins are produced (Fig. 1). All culture conditions were tested for the presence of necrosis-inducing activity by infiltrating culture filtrate into sugar beet leaves (Fig. 1). Ultimately, infiltration of culture filtrate of *C. beticola* grown in Fries medium for seven days, shaking at 120 rpm with exposure to 24 h light caused clear and repeatable necrosis of the host tissue (Fig. 1). Within 24 hours, the host cells within the infiltration area had entirely collapsed while cells outside the area stayed unharmed. Since *C. beticola* is known to produce cercosporin and beticolin that are phytotoxic (26, 27), culture filtrate was treated with a protease mixture to rule out the involvement of phytotoxic secondary metabolites for this necrosis formation. Proteolysis treatment of culture filtrate abolished necrosis formation, confirming that the necrosis-inducing activity can be attributed to a proteinaceous component of the culture filtrate (Fig. 1). To single out the protein responsible for the necrotic phenotype, the active culture filtrate was fractionized using ion exchange chromatography and single fractions were screened for necrosis-inducing activity by individual infiltration into sugar beet leaves (Fig. 1). The fraction that reproducibly caused necrosis was selected for protein identification using MS/MS analysis.

Effector protein candidate identification

Based on the analysis of MS/MS data and subsequent protein identity searches in the *C. beticola* strain 09-40 genome, three candidate effectors were identified: CBET3_03921, CBET3_10646, and CBET3_04765. Of these, CBET3_03921 and CBET3_10646 displayed classic effector

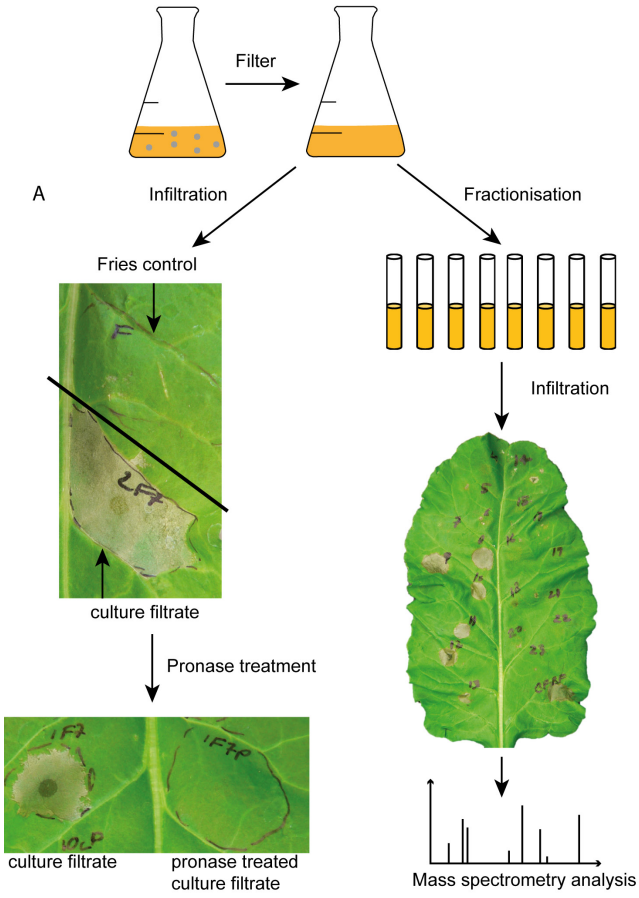

Figure 1. Necrosis-inducing effector identification pipeline. A seven-day old *C. beticola* 09-40 wild-type strain grown in Fries medium was filtered to remove fungal mycelium. (A) When the culture filtrate was infiltrated into seven-week-old sugar beet leaves, a clear necrotic phenotype was observed after 24 h. Proteolysis treatment eliminated necrosis-inducing activity of the culture filtrate. (B) Culture filtrate was fractionated using ion exchange chromatography and necrosis-inducing activity of individual fractions was assayed by infiltration into sugar beet leaves. All infiltration experiments were repeated at least three times using different sugar beet plants.

characteristics including secretion signals, high cysteine content and low molecular weight (9.2 kDa and 6.6 kDa, respectively). In contrast, CBET3_04765 lacked a signal peptide and contained no cysteines and was therefore excluded from further analysis. Interestingly, although CBET3_03921 and CBET3_10646 showed no homology to functionally characterized proteins in the Swiss-Prot database, a large set of homologous, hypothetical proteins was identified for CBET3_03921 when blasted against the NCBI non-redundant database that are mostly derived from Sordariomycetes such as *Fusarium* spp. and *Colletotrichum* spp., and two homologous, hypothetical proteins from *Colletotrichum* spp. were identified for CBET3_10646

when blasted against the same database. The signal peptide cleavage sites were predicted to be between residues 18 and 19 for CBET3_03921 and between 16 and 17 for CBET3_10646 (Fig. 2). Furthermore, the six cysteine residues found in the 85-amino acid sequence of the mature CBET3_03921 protein were predicted to form three disulfide bridges (Fig. 2). Although CBET3_10646 is a rather small protein with 59 amino acids, it is predicted to have four disulfide bonds (Fig. 2). On the nucleotide level, each of the two candidate genes had one intron resulting in a coding sequence of 312 bp for *CBET3_03921* and 228 bp for *CBET3_10646*. While no motifs were detectable for CBET3_03921, CBET3_10646 contains an AxxxG motif that may be involved in dimerization (30). Additionally, a SxxV(K/R) motif, associated with monocation specificity (Cu+, Ag+, and Au+), was also detected (31, 32). While a SxxV(K/R) motif was previously reported to occur in combination with a CxGxxxxDCP metal binding loop, CBET3_10646 appears to only be harboring the monocation specificity domain without the metal binding loop motif.

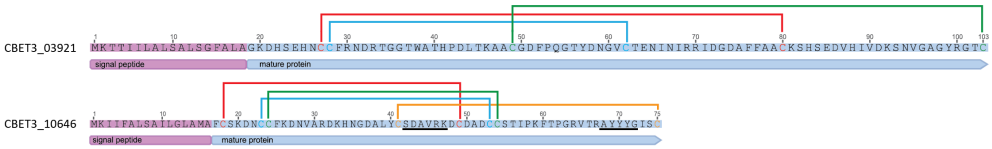

Figure 2. Peptide sequence of CBET3_03921 (CbNip1) and CBET3_10646. Both candidates display classic effector characteristics such as signal peptides and predicted disulfide bonds (highlighted). Underlined in CBET3_10646 sequence are a conserved SxxV(K/R) motif associated with monocation specificity and an AxxxG motif shown to be involved in dimerization.

Heterologous expression of effector protein candidates and phenotype upon infiltration

To further characterize the candidate necrosis-inducing effectors, CBET3_03921 protein was produced heterologously in *Escherichia coli* and infiltrated into sugar beet leaves that were subsequently kept in a growth chamber with a 10-hour light cycle. Unlike the response from the culture filtrate, no phenotype was observed for CBET3_03921 at 24 h (Fig. 3A). However, after 48 h the infiltration area of CBET3_03921 started to appear slightly chlorotic while the empty vector control remained unchanged (Fig. 3 A). Chlorosis of the CBET3_03921 infiltrated area increased over time until it turned necrotic. Since light is critical for the activity of *C. beticola* SM effectors cercosporin and beticolin, we questioned whether light may play a role in the activity of CBET3_03921. To evaluate, we infiltrated CBET3_03921 protein into sugar beet leaves that were subsequently placed in a growth chamber in 24 h darkness. Incubation of CBET3_03921 infiltrated leaves in the dark resulted in clear necrosis of the complete infiltration area by 3 DPI (Fig. 3 B). To assess the stability of CBET3_03921, we incubated the protein and empty vector control at 50°C or 100°C for 30 minutes, after which proteins were infiltrated into sugar beet leaves and subsequently shielded from light exposure. While exposure to 100°C abolished necrosis-formation, samples treated with 50°C were still able to cause necrosis (Fig. 3 C). Furthermore, infiltrations of CBET3_03921 into *Nicotiana benthamiana* led to the same necrotic phenotype, indicating that CBET3_03921 mode-of-action is not host specific (Fig. 3 D). Due to its necrosis-inducing character, we renamed CBET3_03921 to CbNip1 (CbNip for *Cercospora beticola* necrosis-inducing protein 1).

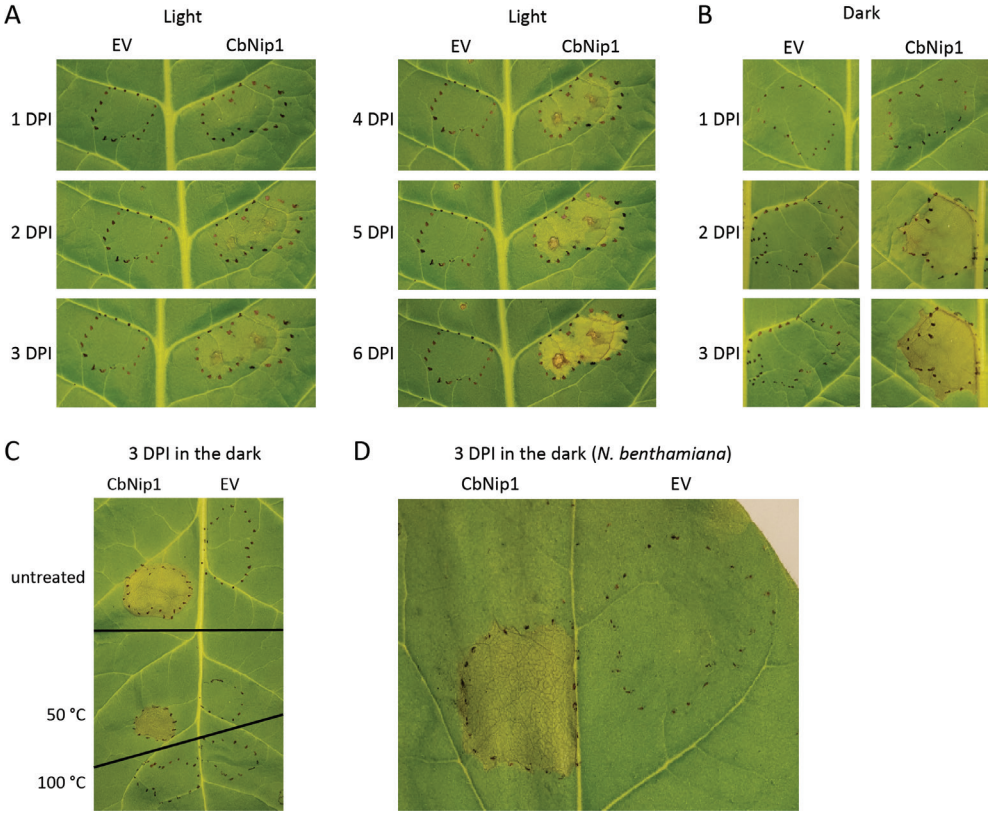

Figure 3. Necrosis-inducing phenotype of CbNip1 protein. (A) Chlorosis/necrosis development after infiltration of CbNip1 into sugar beet leaf exposed to a 10 h/ 14 h light/dark cycle for 6 days, and empty vector sample (EV) infiltration served as a control. (B) Necrosis development after infiltration of CbNip1 into a sugar beet leaf kept in 24 h darkness, and an empty vector infiltration that served as a control. (C) Treatment of CbNip1 and an empty vector exposed to 50°C for 30 min did not affect necrosis-inducing activity of CbNip1 while treatment of both samples at 100°C for 30 min abolished necrosis-induction. Untreated samples served as controls. (D) Necrosis formation after infiltration of CbNip1 into a *N. benthamiana* leaf. An empty vector control sample served a control. All infiltration experiments were repeated at least three times using different sugar beet plants.

We were unable to produce CBET3_10646 in sufficient amounts in either *Pichia pastoris* or *E. coli*. Therefore, chemically synthesized CBET3_10646 protein was used for infiltration into sugar beet leaves. In contrast to CbNip1, no phenotype was visible for the conditions tested, which included light/dark exposure, refolding of the protein and the supplementation of trace elements. Consequently, CBET3_10646 was excluded from further analysis.

In planta gene expression profile of *CbNip1* matches necrotic lesion development

To determine whether *CbNip1* expression pattern during *C. beticola* colonization also matches necrosis emergence *in planta*, we inoculated sugar beet plants with a *C. beticola* wild type

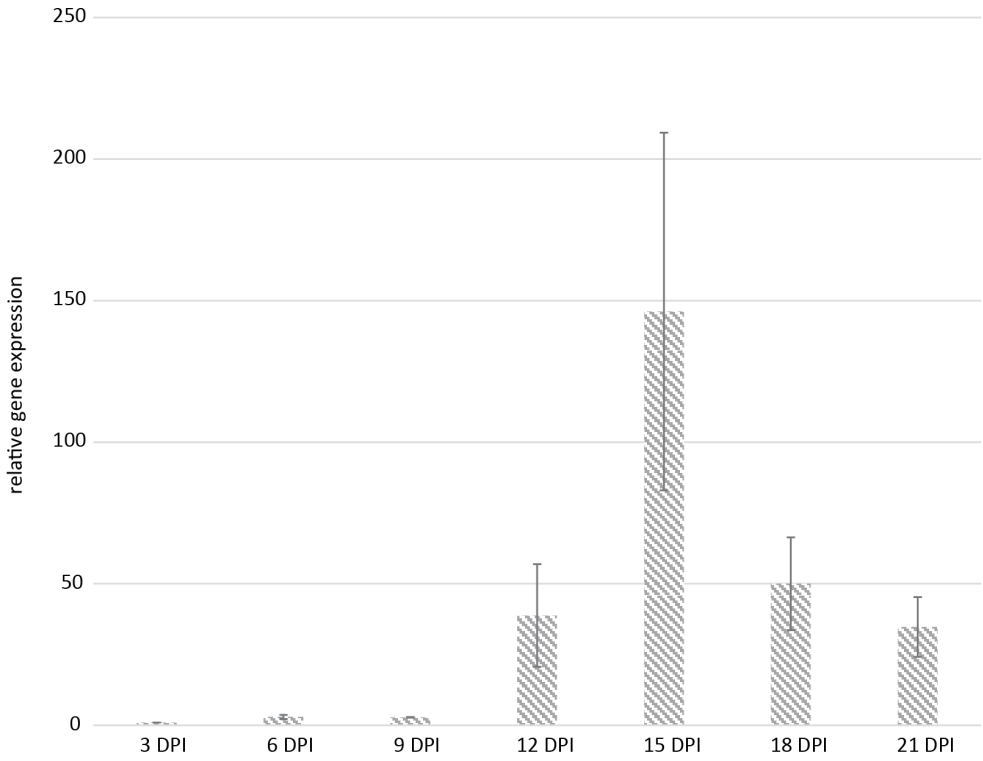

Figure 4. *CbNip1* gene expression during *C. beticola* infection on sugar beet. Gene expression profile of *CbNip1* during 09-40 *C. beticola* wild type strain infection course at 3, 6, 9, 12, 15, 18 and 21 days post infection (DPI). *CbNip1* gene expression was normalized to *C. beticola* actin gene expression. Error bars indicate the standard error of three biological replicates.

strain and harvested leaf samples at 3, 6, 9, 12, 15, 18, and 21 days post inoculation (DPI). Gene expression analysis revealed that *CbNip1* is minimally expressed at early time points (Fig. 4). However, from 12 DPI onwards *CbNip1* expression increased until peaking at 15 DPI. Interestingly onset of *CbNip1* upregulation at 12 DPI matched symptom development on the sugar beet leaves (Suppl. Fig. 2). At 15 DPI, many single necrotic spots were visible while *CbNip1* expression reached its peak. However, with progressing necrosis expansion *in planta*, *CbNip1* experiences a steady downregulation again from 18 DPI onwards (Fig. 4).

CbNip1 is a virulence factor

To investigate whether CbNip1 is required for full *C. beticola* virulence, we inoculated sugar beet plants with wild-type *C. beticola* and gene deletion mutants lacking *CbNip1*. In addition to visible symptom assessment *in planta,* fungal biomass was measured using qPCR for each treatment individually to determine the level of fungal colonization of the host plants. While severe infection

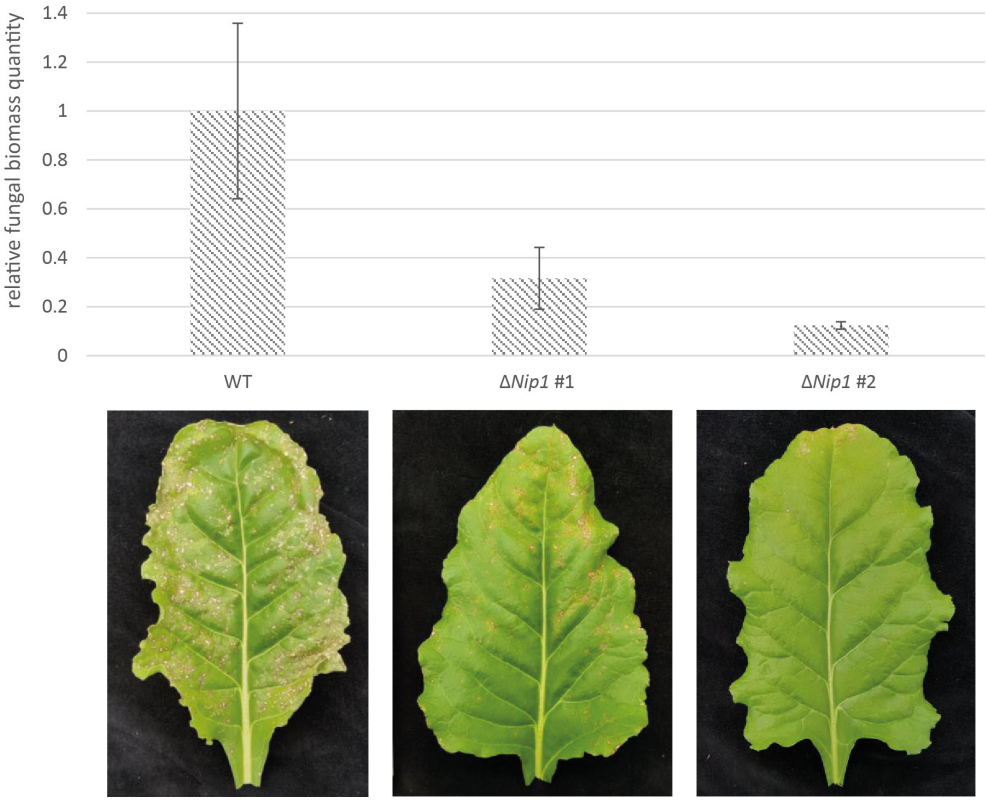

Figure 5. Fungal biomass quantification of *C. beticola* 09-40 wild type strain and two individual Δ*CbNip1* mutants. Sugar beet plants inoculated with *C. beticola* strains at 13 DPI with photos showing respective disease severity below. Error bars represent standard error of five biological replicates.

symptoms were displayed by sugar beet plants inoculated with wild type *C. beticola*, highly-reduced symptom formation was observed for plants inoculated with two individual Δ*CbNip1* strains (Fig. 5). In agreement with the noticeable difference in *in planta* phenotype of wild type and Δ*CbNip1* strains, evaluation of fungal biomass showed reduced fungal colonization in plants infected with Δ*CbNip1* compared to high levels of fungal biomass found in sugar beet plants inoculated with the wild type *C. beticola* strain. In contrast, Δ*CBET3_10646* mutants did not exhibit a virulence penalty when compared with the progenitor wild type *C. beticola* strain (data not shown).

Discussion

C. beticola is a hemi-biotrophic fungus that is dependent on necrosis formation during infection (33) and known to utilize the secondary metabolite effector cercosporin to cause host cell death (26). Here, we report the identification of the novel *C. beticola* necrosis-inducing effector protein CbNip1.

By searching for *in vitro* parameters that trigger *C. beticola* to secrete effector proteins, we found growth conditions under which *C. beticola* produces proteinaceous effectors that cause necrosis upon infiltration into sugar beet leaves within 24 hours. While infiltration of pure CbNip1 into sugar beet leaves took 48 h to lead to visible necrosis, the timing difference in necrosis formation is likely due to the presence of multiple necrosis-inducing effectors besides CbNip1 in the culture filtrate. Interestingly, CbNip1 activity was more pronounced in the dark. Besides CbNip1, fractionation of the culture filtrate with subsequent mass spectrometry analysis of the necrosis-inducing fraction identified the presence of two other proteins CBET3_10646 and CBET3_04765, of which CBET3_04765 was excluded for further analysis due to the lack of typical effector characteristics.

For functional analysis, CbNip1 was heterologously produced and infiltrated into sugar beet leaves. We found that the full potential of CbNip1 to induce host cell death was dependent on the absence of light. Light is known to influence *Cercospora zeae-maydis* infection capability as the ability to find stomata and form appressoria is abolished in the dark (34). Plants are also impacted by light in various ways including alteration of leaf physiology (35, 36). Furthermore, studies on host resistance responses have demonstrated that light is required for the full cascade of plant resistance responses (36-38). Based on micro-array expression profiling of the *C. beticola* - sugar beet interaction, 571 sugar beet genes were induced including pathogenesis-related (PR) genes and genes involved in lignin and alkaloid biosynthesis at the onset of necrotic symptom formation (33). While the products of these defense-associated genes could potentially impede CbNip1 function in the presence of light, PR genes have been shown to be repressed in the dark (36-38). While necrotrophic effectors such as SnTox1 have been reported exploit interactions with defense-associated genes such as specific plant receptors for host cell death induction (11, 14), in case of CbNip1, necrosis is not only induced in sugar beet but also in the non-host *N. benthamiana*. Therefore, it is likely that CbNip1 function may not be sugar beet specific via interaction with a corresponding receptor protein but rather display general toxicity to plants. For example some effectors modulate targets in their host but potentially also in other plants for necrosis induction. This mode-of-action has been observed for the small sRNase Zt6 of *Z. tritici* that displays universal cytotoxicity by cleaving plant and mammalian ribosomal RNA (25). Nevertheless, necrosis formation could also be the result of cell wall degrading enzyme activity as cell wall degrading enzymes of various fungal pathogens have been found to be essential for fungal virulence (39-41).

While a necrotic phenotype is observed for CbNip1, the other effector candidate CBET3_10646 failed to induce any phenotype under tested conditions. Since CBET3_10646 has domains associated with dimerization and monocation specificity, the inability to induce necrosis may be due to the absence of the right cofactor. As CBET3_10646 was chemically synthesized, it is possible that due to its monocation specificity associated domain, the addition of trace elements (including copper) as present in the Fries media of the initial culture filtrate might activate CBET3_10646 function. However, the supplementation of metal ions to CBET3_10646 did not lead to phenotype formation in sugar beet leaves. Moreover, infiltration of CBET3_10646 with CbNip1 did not obviously enhance CbNip1-induced necrosis. Further research is required to identify the allied co-factors for CBET3_10646, if any. Since CBET3_10646 mutants did not exhibit a virulence penalty *in planta*, CBET3_10646 may not be an effector for *C. beticola*.

In accordance with CbNip1 necrosis-inducing ability, we have found that necrotic symptom development *in planta* correlates with up-regulation of *CbNip1* expression (Fig. 4; Suppl. Fig. 2). Induction of host necrosis during the biotrophic phase is not likely beneficial for the fungus, therefore it is not surprising that *CbNip1* is minimally expressed at early infection time points (3 DPI to 9 DPI). An increase in *CbNip1* expression and the development of necrotic lesions occurred simultaneously, suggesting that CbNip1 is linked to the switch from biotrophic to necrotrophic life style of the fungus. Once necrosis formation is ongoing and existing necrotic lesions start to fuse, *CbNip1* expression is reduced again to a similar level as observed in the initial cell death induction phase at 12 DPI, indicating that necrosis induction by CbNip1 may still be important at later time points. Interestingly, *CbNip1* expression pattern is similar to expression patterns of other necrosis-inducing effectors from different protein families found in the hemibiotroph *Z. tritici*. *ZtNIP1* showed an expression pattern where gene upregulation matched onset of symptom development in planta (21). Similarly, the *Z. tritici* Nep-1 like protein *MgNLP* peaked towards the end of the biotrophic phase before necrotic lesions were visible (20). However, there are also examples of contrasting expression patterns to *CbNip1*. For example the expression of *Zt6 in planta* that is characterized by a double peak likely attributed to a double functionality (25).

Since *C. beticola* requires necrotic plant tissue to complete its life cycle (33), we determined whether CbNip1 was also essential for fungal virulence. We found that site-directed *CbNip1* deletion mutants are impeded in virulence compared to the wild type *C. beticola* strain. Not only did plants inoculated with Δ*CbNip1* mutants develop fewer *C. beticola*-specific lesions, biomass determination revealed there was less fungal biomass in plant tissue compared to the progenitor wild type (Fig. 5). Taken together, this indicates that CbNip1 plays an important role in *C. beticola* virulence. As mentioned earlier, *C. beticola* produces the secondary metabolite cercosporin and a family of phytotoxins called beticolins, both of which are able to cause cell death in the presence of light (26, 27, 42, 43) and cercosporin was shown to be a virulence factor for several *Cercospora* species (28, 29). Since light-activation is essential for cercosporin and beticolin functionality, they are likely not active in the dark. With the secretion of CbNip1 however, *C. beticola* may be defying this light-associated limitation by utilizing additional necrosis-inducing agents to cover both light and dark conditions to achieve maximal host cell death to complete its life cycle.

In conclusion, we have shown that *C. beticola* secretes the effector protein CbNip1 during infection that in the absence of light has the ability to cause necrosis upon infiltration into sugar beet leaves within 48 hours. Furthermore, *CbNip1* expression *in planta* correlates with necrotic symptom appearance during *C. beticola* sugar beet infection. Targeted gene replacement of *CbNip1* led to a reduction in virulence, indicating that CbNip1 is a virulence factor for *C. beticola*. As CbNip1 has no obvious homology to other proteins in public databases, future studies will be directed to identify the CbNip1 mode-of-action. Usually, studies on pathogen – host plant interactions focus on processes in the presence of light, however it may be interesting to understand how this interaction is altered in the dark, a vital condition for unhampered CbNip1 function. Consequently, CbNip1 is a fungal virulence factor that is hypothesized to take advantage of the reduced host plant defense response level due to the absence of light. Further analysis of yet unknown functional motifs of CbNip1 as well as localization studies will help to shed light on the biology of CbNip1.

Materials and Methods

Fungal strains

C. beticola wild type strain 09-40 was isolated from leaf material collected from a sugar beet field in the Red River Valley, USA in 2009. The fungus was kept at 22°C on potato dextrose agar (PDA; Difco, Sparks, USA) and fungal site-directed gene deletion mutants in a 09-40 background on PDA amended with 150 µM hygromycin B (Duchefa, Haarlem, NL).

Culture filtrate preparation and infiltration

A 5 mm plug was taken from the actively growing zone of *C. beticola* wild type strain 09-40 on PDA and used to inoculate a 250 ml conical flask filled with 100 ml of Fries media (44). After seven days of incubation at 120 rpm under 24 hour light conditions, the liquid culture was run through two layers of Miracloth (EMS Millipore Corp., Billerica, USA) to clear it of fungal mycelium and subsequently filter-sterilized with a 0.45 µm Filtropur membrane (Sarstedt, Nümbrecht, Germany). Approximately 30 to 50 µl of sterile culture filtrate were infiltrated into the leaves of 7-week-old sugar beet plants of the variety C093 (formerly 86RR66) using a 1 ml needleless syringe. Infiltration experiments were repeated at least three times with multiple individually produced culture filtrates. Plants were kept in a greenhouse chamber with an average temperature of 26°C during the day and approximately 17°C during the night. Chambers were equipped with additional lighting to ensure 16 hours of light a day. To confirm the proteinaceous nature of the necrosis-inducing agent, 50 µl of MOPS buffer (1M, pH 7.5) and 25 µl of pronase (1 mg/ml) (Sigma, St Louis, USA) or water as control was added to 425 µl of culture filtrate and incubated at 22 °C for 4 h. Subsequently, samples were infiltrated into sugar beet leaves as described above.

Culture filtrate fractionation

Culture filtrate was partially purified as described in (45). In short, 100 ml of 7-day-old *C. beticola* wild type strain 09-40 grown in Fries media were first filter-sterilized and then dialyzed against water using a 3.5 kDa molecular weight cutoff dialysis membrane (Fisher Scientific, Pittsburgh, USA). The next day, the dialyzed culture filtrate was loaded onto a HiPrep SPXL 16/10 cation exchange column (GE Healthcare Piscataway, USA) using the ÄKTA prime plus (GE Healthcare, Piscataway, USA) liquid chromatography system. After a washing step with 50 ml of 20 mM sodium acetate buffer pH 5.0, 5 ml fractions were collected during gradient elution of 0 – 300 mM sodium chloride plus 20 mM sodium acetate pH 5.0 at a flow rate of 5.0 ml/min over 20 min. Collected fractions were individually infiltrated into 7-week-old sugar beet plants of the variety C093 (formerly 86RR66) and screened for necrotic phenotype. Fractionation and infiltration experiments were repeated at least three times.

Preparation for MS/MS analysis

The fraction that repeatedly caused necrosis was loaded onto a precast 16.5% tris-tricine polyacrylamide gel (Bio-Rad, Hercules, USA). Protein spots were excised and sent to the Center

for Mass Spectrometry and Proteomics at the University of Minnesota for trypsin digestion and subsequent LCMS analysis. Peptide mass fingerprints and peptide sequence information were used to search for protein identity using the annotated *C. beticola* 09-40 genome (46).

gDNA extraction, RNA extraction, cDNA synthesis

Genomic DNA was extracted using a modified version of the microprep protocol published by Fulton et al. (1995), replacing chloroform:isoamyl alcohol (24:1) with phenol:chloroform:isoamyl alcohol (25:24:1).

RNA extraction followed the Trizol method (Ambion, Carlsbad, USA) according to the manufacturer's protocol and subsequently cleaned up three times using the RNase-Free DNase Set (Qiagen, Hilden, GER) according to Appendix E of the RNase Mini Handbook 06/2012. For cDNA synthesis, 1µg of total RNA was used with the SuperScript III reverse transcriptase kit (Invitrogen, Carlsbad, USA) following the manufacturer's protocol.

Sequence analyses

Signal peptides (if present) were determined with SignalP online tool (ttp://www.cbs.dtu.dk/services/SignalP) while disulfide bonds were predicted using DISULFIND (http://disulfind.dsi.unifi.it/).

RT-PCR of *CbNip1*

Quantitative RT-PCR was performed in triplicate using the SensiMix SYBR Hi-Rox kit (Bioline, Luckenwalde, Germany) with an ABI7300 PCR machine (Applied Biosystems, the Netherlands) and cDNA of each time point for gene expression analysis or gDNA of each treatment for fungal biomass quantification. All reactions were done in triplicate and primers are listed in Suppl. Table S1. Real-time PCR conditions started with a denaturation step of 10 min at 95°C, followed by denaturation for 15 s at 95°C, annealing for 30 s at 60°C, and extension for 30 s at 72°C for 30 cycles. Water as template controls were included for all qPCR runs. With *C. beticola* actin as a reference gene for the gene expression study, relative gene expression of three biological repetitions were calculated in comparison to the earliest measured time point using the Pfaffl method (47). Variation in gene expression was calculated using the standard error of the means of three biological replicates. Biomass was determined using the ΔΔCt method (48) relative to the average value of the wild type inoculated sugar beet plants. Error bars indicate standard error of variation between three individual biological replicates. Primers are listed in Suppl. Table S1.

Vector construction and protein production in E. coli

For heterologous protein expression in *E. coli*, *CbNip1* was amplified with GoTaq Long PCR Master Mix (Promega, Madison, USA) from *C. beticola* 09-40 wild type cDNA using primers MKE-78/77 (Suppl. Table 1), respectively. Amplicons and pET Sumo vector (Invitrogen, Carlsbad, US) were digested with *EcoR*I and *Not*I and followed by ligation of the fragments into the double

digested pET Sumo vector with T4 DNA ligase (NEB, Beverly, USA) and cloned into *E. coli* DH5α. Plasmids carrying the correct *CbNip1* coding sequence were verified by sequencing (Eurofins Genomics, Ebersberg, Germany) and as well as an empty pET Sumo vector subsequently cloned into *E. coli* Origami strain (DE3) strain.

For heterologous protein expression, 1000 ml of LB were inoculated with a 20 ml overnight LB plus kanamycin 50 µg/ml culture with either *CbNip1* pET expression construct or the empty vector control and grown at 37 °C shaking at 200 rpm until reaching an OD_{600} between 0.6-0.8. Protein production was induced with 0.05 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) final concentration and kept growing at 20°C shaking at 200 rpm for 24 hours. Cells were pelleted, snap frozen with liquid nitrogen and then lysed with 20 ml lysis buffer containing 50 mM Tris-HCL pH 8.5 (Invitrogen, Carlsbad, USA)and 150 mM NaCl (Sigma, St Louis, USA), 10% glycerol (Amresco, Solon, USA), 6 mg/ml lysozyme from chicken egg white (Sigma, St Louis, USA), 2 mg/ml sodium deoxycholate (Sigma, St Louis, USA), 0.625 mg/ml Deoxyribunuclease I from bovine pancreas (Sigma, St. Louis, USA), and one cOmplete protease inhibitor pill (Sigma, Mannheim, Germany). After the cultures were kept on ice for 1½ hours, cells debris was spun down for one hour at 14000 rpm at 4°C and the soluble protein fraction was processed for protein purification.

Protein purification

In *E. coli* heterologously produced protein samples were loaded at 1 ml/min onto a column packed with 2ml of Ni Superflow Resin (Clontech, Mountain View, US) for purification. After a washing step with wash buffer (50 mM Na2HPO4 (Merck, Darmstadt, Germany), 300 mM NaCl (Sigma, St Louis, USA), 40 mM imidazole (Merck, Darmstadt, Germany) at 2 ml/min to wash out contaminative *E. coli* native proteins, SUMO-tagged CbNip1 or the Sumo tag alone obtained from the empty vector sample were eluted with elution buffer 50 mM Na2HPO4 (Merck, Darmstadt, Germany), 300 mM NaCl (Sigma, St Louis, USA), 40 mM imidazole (Merck, Darmstadt, Germany). Elution samples were dialyzed with a Spectra/Por Dialysis Membrane with MWCO of 3,500 (Spectrum Laboratories, Rancho Dominguez, USA) against 200 mM NaCl containing ULP-1 enzyme to cleave off the SUMO tag at 4 °C overnight without agitation. The next day samples were run through the Nickle bead column with the same setup as before at 1 ml/min to allow cleaved off SUMO tags to bind to the Nickle beads. Flow-through was collected and again dialyzed for 24 hours against 200 mM NaCl. Samples were concentrated with Amicon Ultra-15 centrifugal filter unit with an Ultracel-3 membrane (Millipore, Billerica, USA) with a 3 kDa cut off. For visualization, five µl of protein sample were loaded on Mini-PROTEAN TGX stain free precast gels (Biorad, Hercules, USA).

Refolding and preparation of CBET3_10646 for sugar beet leaf infiltration

Synthesized mature CBET3_10646 purchased from GeneScript (Piscataway, USA) was dissolved in MQ water to 3 mg/ml. For refolding, oxidized glutathione (Sigma, St Louis, USA) and reduced glutathione (Sigma, St Louis, USA) were added to 1 mg/ml of CBET3_10646 to an end ratio of 5:1 mM and incubated overnight. MQ water treated with the same glutathione ratio served as a control. To see whether the addition of trace elements leads to activation of necrosis-inducing

activity of CBET3_10646, 1 µl of trace element stock were added to 0.5 ml of protein sample or water control to a trace element end concentration as found in Fries media used for the culture filtrate experiment.

Protein infiltration

Sugar beet of the variety C093 (formerly 86RR66) were grown in the climate chamber at 21°C with 10 hours light with 10 lux and 70 % humidity. After 7 weeks approximately 30 to 50 µl of purified protein (~2 mg ml-1) or empty vector (Suppl. Fig. 1) were infiltrated into the leaves using a 1 ml needleless syringe and the infiltration area was marked with a marker. Dark-treated leaves were wrapped in aluminum foil to prevent light exposure. For this experiment, CBET3_10646 and three individually produced and purified CbNip1 samples were infiltrated at least three times.

Deletion mutants

Site-directed gene deletion mutants were generated following the split-marker PEG-protocol described in Bolton *et al*. (2016) (49). Primers are listed in Suppl. Table S1. Gene deletion was verified with two different approaches, by absence of PCR product using gene specific forward and revere primers and by presence of an amplicon for a forward primer designed upstream of the 5' flanking region of the target gene and a reverse primer annealing to the hygromycin resistance cassette.

Inoculation assay

Spore formation of *C. beticola* wild type and two individual deletion mutants was induced on CV8 agar plates as previously described (50). Spores were harvested and adjusted to a concentration of 1 × 105 spores per ml and spore suspension was equally spayed on the lower sides of the leaves of 7-week-old sugar beet plants of the variety C093 (formerly 86RR66). Inoculated plants were kept in a humidity tent inside the greenhouse chamber with about 27°C and 90 % humidity for 5 days after which the tent was removed and plants were exposed to 22°C with a 16-h/8-h day/night cycle. For fungal biomass analysis three leaves of two plants for five repetitions were harvested at 13 DPI (days post infection) and instantly snap frozen while plants for gene expression analysis were harvested 3, 6, 9, 12, 15, 18, and 21 DPI using three leaves of two plants in three repetitions.

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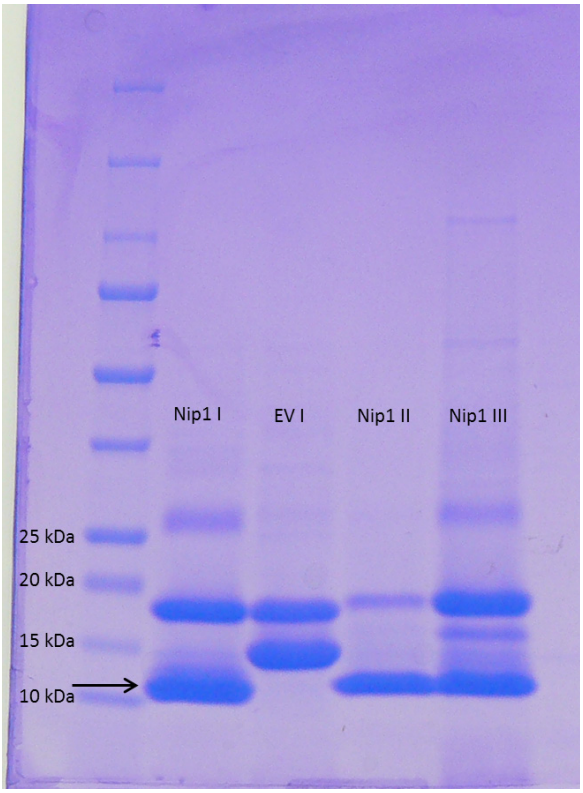
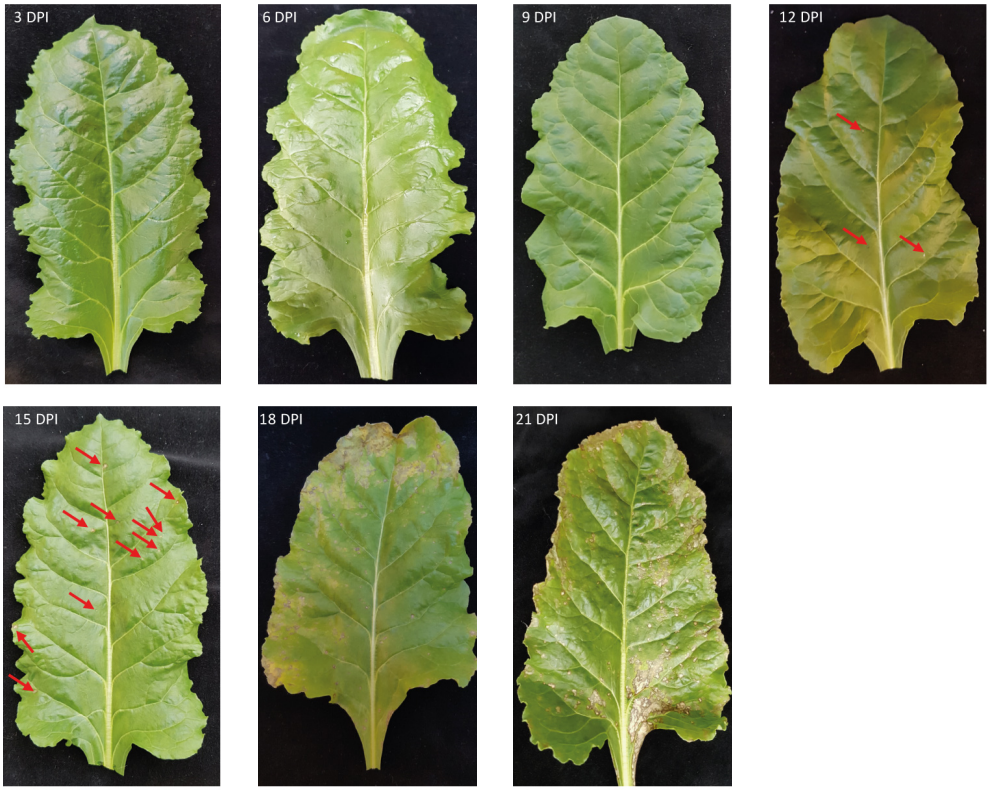
Supplementary data



Supplementary Figure 1. Gel visualization of infiltrated CbNip1 protein and empty vector samples. SDS-PAGE gel loaded with 5 µl of heterologously expressed protein sample used for infiltration studies. Nip1 I, Nip1 II, and Nip1 III are three individually produced and purified CbNip1 protein samples (arrow indicates CbNip1 protein). EVI is the individually produced and purified empty vector sample used as control.



Supplementary Figure 2. Symptom development during *C. beticola* 09-40 wild type strain infection of sugar beet. No symptoms are detectable at early time points (3, 6, and 9 DPI). Red arrows at 12 and 15 DPI indicate formation of necrotic lesions. At 18 DPI, necrotic lesion formation intensifies, accompanied by some chlorosis around the necrotic lesions. At 21 DPI, single necrotic lesions are fused to form necrotic patches on the sugar beet leaf.

Table S1. Primers used in this study.

Primer	Sequence (5'- 3')	Description
MKE-78	C G G T A T G A A T T C GGCAAAGACCACTCCGAGCAC	CbNip1 Fp with EcoRI RE site for insertion into pET
MKE-77	CGTCTAGCGGCCGCCTACTACTA GCAAGTTCCACGGTAACCCGC	CbNip1 Rp with triple stop codon and NotI RE site for insertion into pET
MDB-726	ACTTGCCTGGCTTTTGTTTCTAGT	SbEc1-F qPCR for sugar beet biomass
MDB-727	GCCAGGTGCTGACTTGATTATTT	SbEc1-R qPCR for sugar beet biomass
MDB-284	ACATGGCTGGTCGTGATTTG	C.beticola actin qPCR Fp
MDB-285	TGTCCGTCAGGAAGCTCGTA	C.beticola actin qPCR Rp
MDB-1063	AGACCACTCCGAGCACAACT	CbNip1 qPCR Fp
MDB-1064	ACACCGTTGTCGTAGGTTCC	CbNip1 qPCR Rp
MDB-957	CCTGTGGTCTGAGCTTGTCA	CbNip1 1F for KO
MDB-958	CACTGGCCGTCGTTTTACAACGTC TCCAACTGTTCTCCCTGTCC	CbNip1 2R for KO
MDB-959	TCATGGTCATAGCTGTTTCCTGTG GGTTGTTGGGGAGTTTCTGA	CbNip1 3F for KO
MDB-960	CACCACTTGGTATCGGGAAC	CbNip1 4R for KO
MDB-1541	AGCCGCTAATCACCCAAGAT	CbNip1 5p1F
MDB-277	GACGTTGTAAAACGACGGCCAGTG	Split-marker HYG-F
MDB-258	GGATGCCTCCGCTCGAAGTA	Split-marker HY-R
MDB-259	CGTTGCAAGACCTGCCTGAA	Split-marker YG-F
MDB-278	CACAGGAAACAGCTATGACCATGA	Split-marker HYG-R
MDB-1145	GGCAGGTAGATGACGACCAT	HYG R2

Chapter 5

Gene cluster conservation provides insight into cercosporin biosynthesis and extends production to the genus *Colletotrichum*

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Abstract

Species in the genus *Cercospora* cause economically devastating diseases in sugar beet, maize, rice, soy bean and other major food crops. Here we sequenced the genome of the sugar beet pathogen *C. beticola* and found it encodes 63 putative secondary metabolite gene clusters, including the cercosporin toxin biosynthesis (*CTB*) cluster. We show that the *CTB* gene cluster has experienced multiple duplications and horizontal transfers across a spectrum of plant pathogenic fungi, including the wide-host range *Colletotrichum* genus as well as the rice pathogen *Magnaporthe oryzae*. Although cercosporin biosynthesis has been thought to-date to rely on an eight gene *CTB* cluster, our phylogenomic analysis revealed gene collinearity adjacent to the established cluster in all *CTB* cluster-harboring species. We demonstrate that the *CTB* cluster is larger than previously recognized and includes *cercosporin facilitator protein* (*CFP*), previously shown to be involved with cercosporin auto-resistance, and four additional genes required for cercosporin biosynthesis, including the final pathway enzymes that install the unusual cercosporin methylenedioxy bridge. Finally, we demonstrate production of cercosporin by *Colletotrichum fioriniae*, the first known cercosporin producer within this agriculturally important genus. Thus, our results provide new insight into the intricate evolution and biology of a toxin critical to agriculture and broaden the production of cercosporin to another fungal genus containing many plant pathogens of important crops worldwide.

Introduction

Cercospora are among the most speciose genera in all Fungi (1). First described in 1863 (2), the genus has sustained a long history, largely due to notoriety as the causal agent of leaf spot diseases in a wide range of plants including agriculturally important crops such as sugar beet, soybean, maize, and rice that together account for hundreds of millions of dollars in lost revenue annually to growers worldwide (3-8). Although *Cercospora* spp. share several characteristics associated with pathogenicity, such as penetration through natural openings and extracellular growth during the biotrophic stage of infection, most rely on the production of the secondary metabolite (SM) cercosporin (1) to facilitate infection (9, 10). Studies spanning nearly 60 years have made cercosporin a model perylenequinone (11), a class of SMs characterized by a core pentacyclic conjugated chromophore that gives rise to its photoactivity. When exposed to ambient light, cercosporin is a potent producer of reactive oxygen species in the presence of oxygen (12) with a quantum efficiency of >80% (13). This small molecule is lipophilic and can readily penetrate plant leaves leading to indiscriminate cellular damage within minutes of exposure (14). Indeed, cercosporin is nearly universally toxic to a wide array of organisms including bacteria, mammals, plants, and most fungal species with the key exception of cercosporin-producing fungi, which exhibit cercosporin auto-resistance. To date, cercosporin has only been reported to be produced by *Cercospora* spp., with the single exception of the brassica pathogen *Pseudocercosporella capsellae* (15). However, *Pseudocercosporella* and *Cercospora* are phylogenetically closely related, residing in a large clade within the Mycosphaerellaceae (16).

In contrast to the large body of information on cercosporin biology spanning several decades (17, 18), the cercosporin toxin biosynthesis (*CTB*) gene cluster was only recently resolved in *C. nicotianae* (19). The keystone enzyme for cercosporin biosynthesis, CTB1, bears all the hallmarks of an iterative, non-reducing polyketide synthase (NR-PKS) (20). Using *CTB1* as a point of reference, the complete *C. nicotianae CTB* gene cluster was determined to consist of eight contiguous genes of which six are believed to be responsible for cercosporin assembly (*CTB1*, *2*, *3*, *5*, *6*, and *7*) (19, 21). The zinc finger transcription factor CTB8 co-regulates expression of the cluster (19), while the major facilitator superfamily (MFS) transporter CTB4 exports the final metabolite (22). Downstream of the *CTB* cluster are two open reading frames (ORFs) encoding truncated transcription factors, while loci designated as *ORF9* and *ORF10* upstream of the *CTB* cluster are not regulated by light and are not believed to encode proteins with metabolic functions (19). Consequently, the clustering of eight genes with demonstrated co-regulation by light that are flanked by ORFs with no apparent role in cercosporin biosynthesis has suggested that cercosporin production relies on the eight-gene *CTB* cluster (19). In this study, we used an evolutionary comparative genomics approach to show that the *CTB* gene cluster underwent multiple duplication events and was transferred horizontally across large taxonomic distances. Since these horizontal transfer events included genes adjacent to the canonical eight gene *CTB* cluster, we used reverse genetics to show that the *CTB* cluster includes additional genes in *C. beticola*, including one gene that was previously shown to be involved with cercosporin auto-resistance (23) and four previously unrecognized genes involved with biosynthesis. The *CTB* cluster was found in several *Colletotrichum* (*Co.*) species, and we confirmed that the apple pathogen *Co. fioriniae*

can also produce cercosporin. As all earlier understanding of cercosporin biosynthesis has been unwittingly limited by a truncated set of genes in *Cercospora* spp., the full dimension of the gene cluster provides deeper insight into the evolution, biosynthesis and dissemination of a fungal toxin critical to world-wide agriculture.

Results

Secondary metabolite cluster expansion in Cercospora beticola.

C. beticola strain 09-40 was sequenced to 100-fold coverage and scaffolded with optical and genome maps, resulting in 96.5% of the 37.06 Mbp assembly being placed in 12 supercontigs of which 10 are assumed to be chromosomes. Despite their ubiquitous presence in nature and cropping systems, genome sequences of *Cercospora* spp. are not well-represented in public databases. Therefore, to aid comparative analysis within the *Cercospora* genus we also sequenced the genome of *C. berteroae* and reassembled the genome of *C. canescens* (24) (Suppl. Table S1). To identify gene clusters responsible for biosynthesis of aromatic polyketides in *C. beticola,* we mined the genome to identify all SM clusters (25) and compared these with predicted clusters in related Dothideomycete fungi. The *C. beticola* genome possesses a total of 63 predicted SM clusters of several classes, representing an expanded SM repertoire with almost twice the number when compared to closely related Dothideomycetes, which average 34 SM clusters (Suppl. Table S2; Dataset S1). Notably, *C. beticola* encodes 23 candidate non-ribosomal peptide synthetase (NRPS) clusters, which is considerably higher than most Dothideomycetes, which have an average of 13 (26). To identify the *C. beticola* PKS cluster responsible for cercosporin biosynthesis, we compared the sequence of the *C. nicotianae CTB* cluster (19) with predicted PKS clusters of *C. beticola*. To fill in sequencing gaps between genes in the *C. nicotianae CTB* cluster, we sequenced the genome of *C. nicotianae*, which showed that *C. beticola* PKS *CBET3_00833* (*CbCTB1*) and flanking genes (*CBET3_00830 – CBET3_00837*) were ~96% identical to *C. nicotianae CTB1 – CTB8* and all genes were collinear, strongly suggesting this region houses the *CTB* cluster in *C. beticola* (Suppl. Fig. S1).

Repeated duplication and lateral transfer of the cercosporin biosynthetic cluster.

To study the evolutionary relationships of *C. beticola* PKSs, we conducted large-scale phylogenomic analyses that included various previously characterized PKSs from selected species (27). Since resolving orthologous relationships among PKSs can predict the type of SM that will be synthesized, we first built a phylogenetic tree of the conserved core β-ketoacyl synthase (KS) domains of each PKS that resulted in separating PKS enzymes into four major groups (Suppl. Fig. S2A). Among the eight *C. beticola* NR-PKSs, phylogenetic analysis revealed significant similarity between *CbCTB1*, *CBET3_10910-RA*, and *CBET3_11350-RA* which cluster at the base of the cercosporin clade (Suppl. Fig. S2B). Interestingly, genes flanking *CBET3_10910-RA*, but not *CBET3_11350-RA*, were also strikingly similar to *CbCTB* cluster genes (Fig. 1). Consequently, we hypothesize that the *CBET3_10910* SM cluster is the result of a *CTB* cluster duplication. Since duplicated SM gene clusters appeared to be relatively rare in fungi (28), we investigated the origin and specificity of the *CTB* cluster and the putative duplication by searching for *CbCTB1* homologs against a selected set of 48 published Ascomycete proteomes (Suppl. Table S3) representing a

diverse group of fungal orders. We identified *Cb*CTB1 orthologs in *Cercospora* spp. *C. berteroae* and *C. canescens* and confirmed its presence in *Cladosporium fulvum* (27) and *Parastagonospora nodorum* (29). Surprisingly, seven additional orthologs were identified in Sordariomycete species *Co. orbiculare*, *Co. gloeosporioides*, *Co. fioriniae*, *Co. graminicola*, *Co. higginsianum*, and *Magnaporthe oryzae* as well as one in the Leotiomycete *Sclerotinia sclerotiorum* (Suppl. Fig. S3*A*), representing diverse taxa harboring *CTB1*. Analysis of sequence identity showed that intra-species (*Cb*CTB1 – CBET3_10910-RA) sequence identity (45%) was lower than the inter-species identity (e.g. *Cb*CTB1 and *C. fulvum* CTB1 (Clafu1_196875) sequence identity is 55%; Suppl. Table S4), suggesting that the *CTB1* duplication event occurred prior to Dothideomycete speciation.

To develop a 'phylogenetic roadmap' that may explain *CTB1* evolution, we used the process of 'reconciliation' that takes into account both species and gene histories (30). Although not conclusive, reconciliation considers the costs of evolutionary events (i.e. gene duplications, transfers, and/or losses) to explain the most parsimonious evolutionary route to the present scenario (31). Reconciliation of the species tree (Suppl. Fig. S4) with the CTB1 protein tree revealed that the predicted evolutionary history of CTB1 can be characterized by four duplications, three transfers, and widespread loss to most species analyzed (Suppl. Fig. S5*A*), and further corroborates our hypothesis that the *CTB1* duplication event (D1) occurred early in Dothideomycete speciation. Reconciliation also revealed an ancient *CTB1* ortholog in *S. sclerotiorum* (Suppl. Fig. S5*A*), suggesting that *CTB1* arose prior to speciation of Dothideomycetes. Duplications 2-4 (D2-4) arose after lateral transfer (T1) of *CTB1* into the last common ancestor of the Glomerellales. *CTB1* was then transferred (T2) from a common ancestor in the *Glomerellales* to *M. oryzae* (Suppl. Fig. S5*A*).

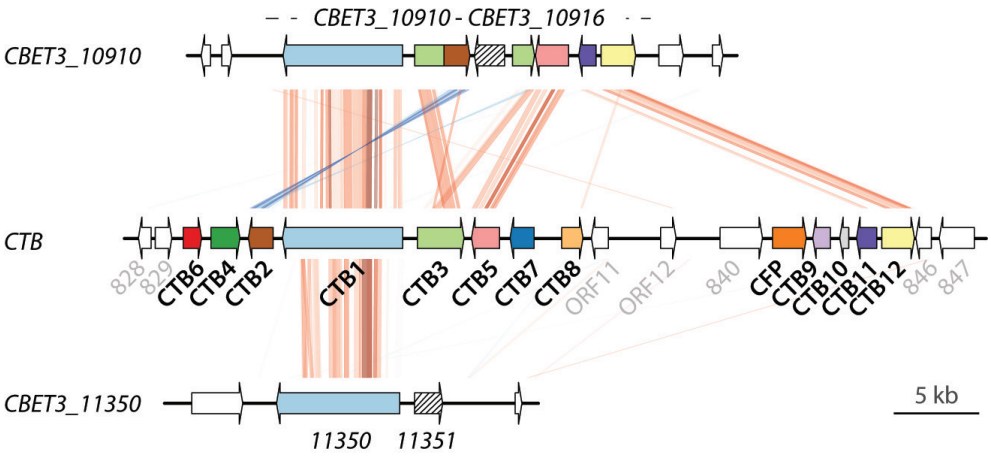

Figure 1. The cercosporin biosynthetic cluster is duplicated and maintained in *C. beticola*. *CBET3_10910* and flanking genes are syntenic with the *CTB* cluster (*CBET3_00833* and flanking genes) in *C. beticola*. Alignment lines correspond to DNA fragments exhibiting significant similarity when the genomic regions comprising the gene clusters are compared with tBLASTx. Direct hits are displayed in red, whereas complementary hits are in blue. The intensity of the alignments represents the percentage similarity ranging from 23 to 100 percent. Genes flanking *CBET3_11350-RA* were not syntenic with *CTB* cluster genes.

We extended the search for CTB cluster protein orthologs by scanning the 48 proteomes for homologs of *Cb*CTB2 (CBET3_00830) to *Cb*CTB8 (CBET3_00837) followed by phylogenetic tree construction and subtree selection (Suppl. Fig. S3*B-N*). This resulted in the identification of orthologs in the same set of species previously listed to contain *CTB1*, with the only exceptions in cases where *CTB* gene homologs were lost in a species. Although the loss of CTB6 and CTB7 orthologs limits reconciliation analysis of these gene families, reconciliation of the subtrees for CTB2, CTB3, CTB4, CTB5, and CTB8 (Suppl. Fig. S5*B-H*) supported a similar scenario as proposed for *CTB1*, involving at least two duplications (D1 and D2) and two horizontal transfer events (T1 and T2) that explain the present-day *CTB* scenario (Fig. 2). However, an alternative explanation involving a single transfer to an ancestral Glomerellales species followed by widespread loss in most species in this lineage except for *M. oryzae* and the analyzed *Colletotrichum* spp. (Fig. 2; Suppl. Table S5) cannot be ruled out by our analyses at this stage.

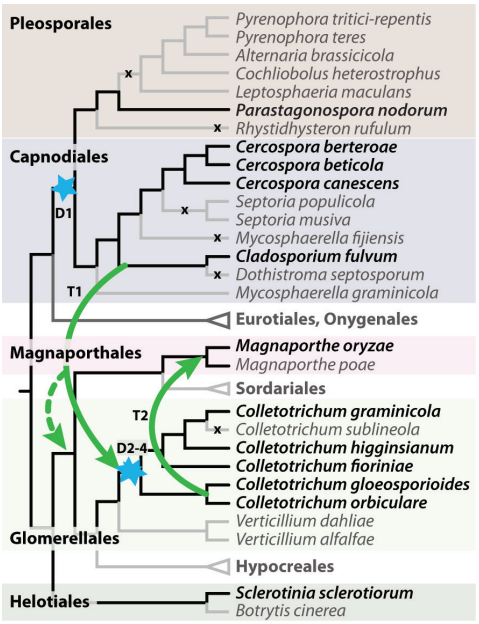

Figure 2. Phylogenetic roadmap of CTB cluster evolution. Phylogenetic roadmap detailing the proposed evolutionary trajectory of the *CTB* cluster involving horizontal gene transfer events from the Capnodiales to the Glomerellales (T1) and another from the Glomerellales to the Magnaporthales (T2) as well as multiple duplications (D1-4) and frequent gene loss (x). Cladogram of the phylogenetic relationship of *Cercospora* spp. and 45 other sequenced fungi. The unscaled tree was constructed using CVTree. Duplication nodes are marked with blue stars, losses are indicated by the crosses and transfers are highlighted by green arrows. Species without the *CTB* cluster are depicted in grey, those encompassing it are in black. An alternative and slightly less parsimonious scenario involving a single transfer from Capnodiales into the last common ancestor of the Magnaporthales and the Glomerellales is shown by the dashed arrow.

Extension of the predicted cercosporin biosynthetic cluster based on microsynteny.

To further examine the *CTB* clusters across all recipient species we generated pairwise alignments relative to the *C. beticola CTB* cluster and flanks. To our surprise, we observed a striking level of similarity outside of the known eight *CTB* genes on the 3' end of the cluster (Fig. 3) in all *CTB*-containing genomes. To investigate whether the amount of microsynteny observed for *CTB* cluster and these flanking genes can be reasonably expected when comparing Dothideomycete and Sordariomycete genomes, we assessed the genome-wide microsynteny between the genomes of *C. beticola* and *Co. gloeosporioides* and *C. beticola* and *M. oryzae*. This analysis identified the *CTB* cluster together with its flanking genes as having

the highest level of microsynteny among all regions in the genome between *C. beticola* and *Co. gloeosporioides*, and showed that the observed *CTB* microsynteny between *C. beticola* and *M. oryzae* was also higher than the genome-wide average (Fig. 4). Likewise, sequence identity of most CTB proteins between *C. beticola* and *Colletotrichum* spp., and to a lesser degree with *M. oryzae*, is higher compared to the genome-wide average (Suppl. Fig. S6, S7). In contrast, sequence conservation of CTB8, a Zn_2Cys_6 transcription factor previously implicated for transcriptional regulation of the *CTB* cluster (19), appears much lower than that of other CTB- and non-CTB proteins, and therefore is suggestive of positive diversifying selection. Considering the level of microsynteny and protein conservation, we hypothesized that these

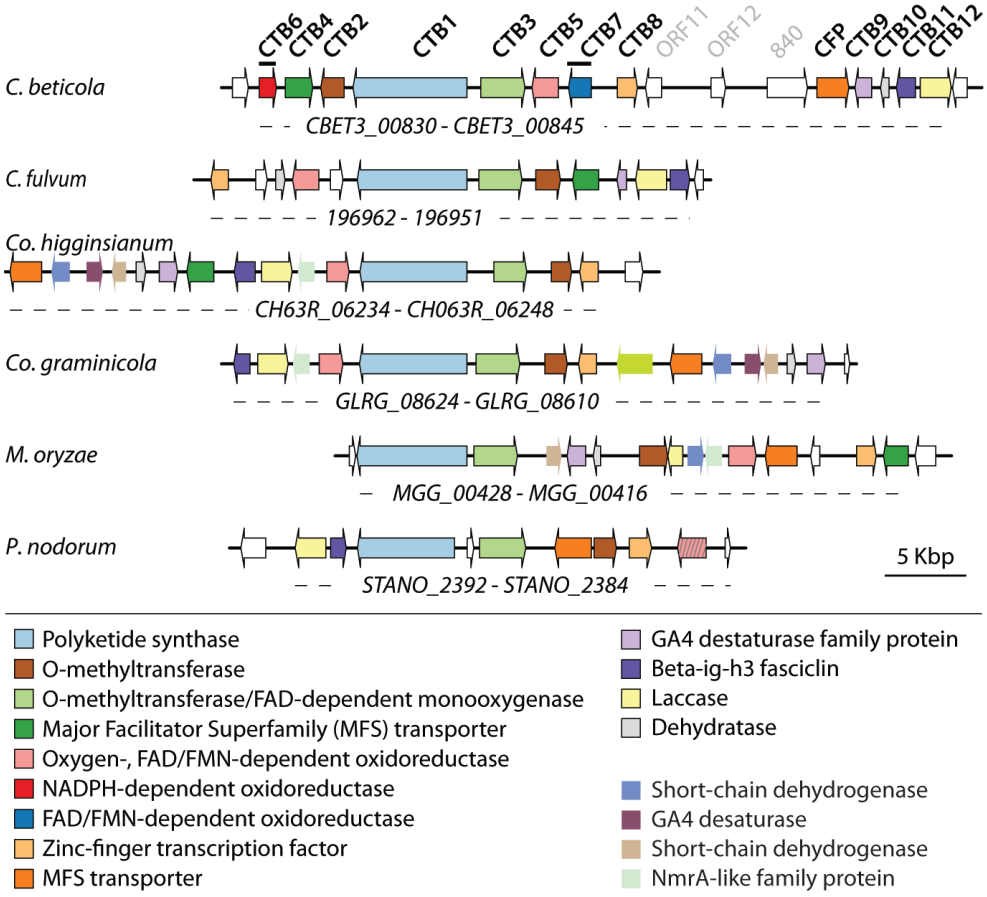

Figure 3. Synteny and rearrangements of the conserved *C. beticola* cercosporin biosynthetic cluster. The cercosporin biosynthetic cluster in *C. beticola* (Cb), top line, and flanking genes are conserved in *Cladosporium fulvum*, *Co. higginsianum*, *Co. graminicola*, *M. oryzae* and *Parastagonospora nodorum*. For all species, the displayed identifiers are transcript IDs and the corresponding sequences can be retrieved from JGI MycoCosm or ORCAE. *CTB* orthologs are colored relative to the *C. beticola CTB* cluster genes and the color key, as well as annotated functions, are highlighted below the *CTB* cluster graphic. *Cercospora*-specific *CTB* genes *CTB6* and *CTB7* are underlined.

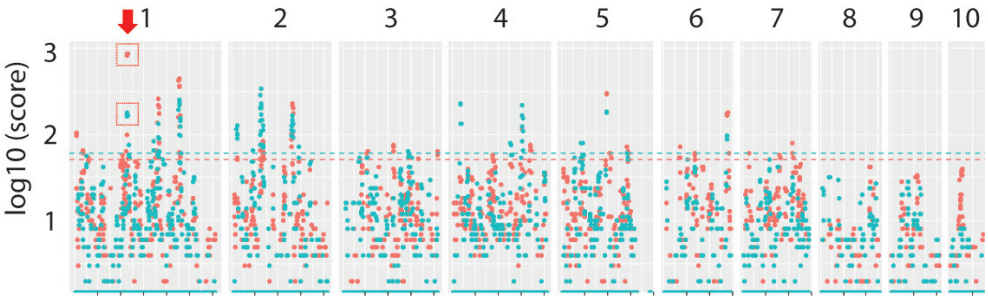

Figure 4. *CTB* cluster microsynteny conservation segregates from the genome-wide average. The genome-wide, gene-by-gene microsynteny between *Cercospora beticola* and *Colletotrichum gloeosporioides* (Cg, red), and between *C. beticola* and *M. oryzae* (Mo, blue), across the ten assembled *C. beticola* chromosomes is shown. Each dot represents one *C. beticola* gene and its respective microsynteny score. The red arrow indicates the position of the *CTB* cluster on chromosome 1 and coincides with high microsynteny in both *Co. gloeosporioides* and *M. oryzae*. The dashed lines represent the 99th quantile of the microsynteny scores for both comparisons independently.

flanking genes are part of the *C. beticola CTB* cluster. To test this proposal, we first determined the relative expression of all eight established *C. beticola CTB* genes as well as a number of flanking genes (*CBET3_00828* to *CBET3_00848*) under light (cercosporin-inducing) compared to dark (cercosporin-repressing) conditions, which showed that all candidate *CTB* genes on the 3' flank were induced in the light except *CBET3_00846* and *CBET3_00848* (Suppl. Table S7). Functional annotation of these genes revealed one non-conserved phenylalanine ammonia lyase (CBET3_00840), the cercosporin facilitator protein (CFP) (23) (CBET3_00841), a candidate a-ketoglutarate-dependent dioxygenase (CBET3_00842), a dehydratase (CBET3_00843), a β-ig-h3 fasciclin (CBET3_00844), a laccase (CBET3_00845), zinc finger domain-containing protein (CBET3_00846), and protein phosphatase 2A (CBET3_00847; Suppl. Table S7), several of which have functions associated with multi-domain enzymes or polyketide biosynthesis in fungi or bacteria (19, 32-37). Phylogenetic analyses of these flanking genes and reconciliation of their respective protein phylogenies (Suppl. Fig. S3, S5) with the species tree (Suppl. Fig. S4) suggest that all genes except *CBET3_00840*, *CBET3_00846*, and *CBET3_00847* have undergone highly similar evolutionary trajectories as the established *CTB* cluster genes (Fig. 2, Suppl. Fig. S5) suggesting that the CTB cluster was transferred as a whole at least once, followed by species-specific evolutionary trajectories involving frequent gene loss as well as gene gain (Fig. 2). We further evaluated the hypothesis of horizontal cluster transfer using a comparative topology test that examines whether the determined tree topologies that support horizontal cluster transfer are significantly better than constrained topologies that would not support transfer. Tree topologies were compared using the Approximately Unbiased test (38), implemented in CONSEL (39) as previously described by Wisecaver and Rokas (2015) (40). Constrained topologies in which we force either a monophyletic origin of all Dothideomycete proteins or a monophyletic origin of all Sordariomycete proteins were significantly worse than trees without such constraint (Suppl. Table S6). Thus, the comparative topology tests support the previously determined topologies, which suggest horizontal cluster transfer.

Novel CTB genes are essential for cercosporin biosynthesis.

To confirm individual gene contributions for cercosporin production, we generated single gene deletion mutants of all candidate genes from *CBET3_00840* to *CBET3_00846* and tested their ability to produce cercosporin. Initial assays of selected mutants showed that cercosporin production in Δ*CBET3_00844* and Δ*CBET3_00845* mutants was abolished, while Δ*CBET3_00842* mutants accumulated only a red, cercosporin-like metabolite that migrated differently in potato dextrose agar (PDA) culture plates and thin layer chromatography (TLC) (Suppl. Fig. S8). To provide more definitive analyses of cercosporin production, high-performance liquid chromatography (HPLC) profiles were obtained from all candidate *CTB* gene mutants and compared to WT cercosporin (Fig. 5A). Unlike other analyzed mutants, Δ*CBET3_00840* and Δ*CBET3_00846* produced compounds with HPLC profiles like cercosporin (Fig. 5A), suggesting these genes are not involved with cercosporin biosynthesis. Taken together, these results corroborate our hypothesis that the *CTB*

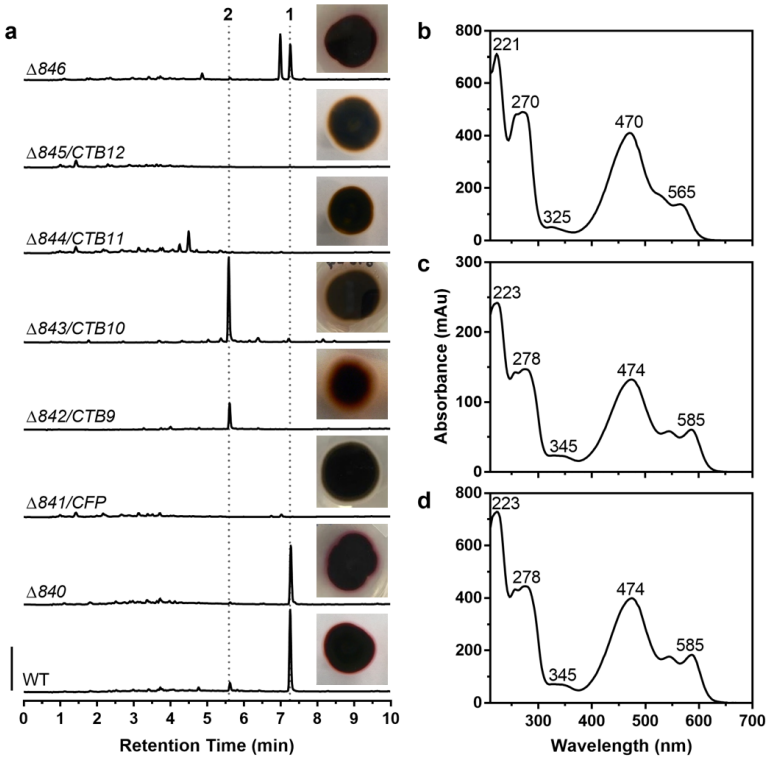

Figure 5. Analysis of cercosporin production in CTB mutants of *C. beticola*. Site-directed knock-out mutants in genes *CBET3_00840*, *CFP* (*CBET3_00841*), *CTB9* (*CBET3_00842*), *CTB10* (*CBET3_00843*), *CTB11* (*CBET3_00844*), *CTB12* (*CBET3_00845*) and *CBET3_00846* were assayed for cercosporin production by HPLC. Cercosporin extracted from *C. beticola* strain 10-73-4 (WT) was used as a positive control. **a)** 280 nm HPLC chromatograms and images of representative colonies for each knock-out. Scale bar indicates 250 mAu. Cercosporin (**1**) and pre-cercosporin (**2**) peaks are indicated by dashed lines. **b-d)** UV-Vis spectra from wild-type *C. beticola* (**b**, 7.25 min peak), *C. beticola* Δ*CTB9* (**c**, 5.36 min peak), and *C. beticola* Δ*CTB10* (**d**, 5.36 min peak) were extracted from 280 nm HPLC chromatograms. Wavelengths of relevant UV maxima are indicated.

cluster extends to at least *CBET3_00845* at the 3' side and includes four additional *CTB* biosynthetic genes as well as *CbCFP*. Consequently, we propose naming genes *CBET3_00842*, *CBET3_00843*, *CBET3_00844* and *CBET_00845* as *CTB9* to *CTB12*, respectively (Suppl. Table S7).

Pre-cercosporin isolation and characterization.

To characterize the red metabolite that accumulated in the Δ*842/CTB9* and Δ*843/CTB10* mutants (Fig. 5A; (Suppl. Fig. S8), an ethyl acetate extract of the collected mycelia was analyzed by reverse-phase HPLC. At 280 nm, a single peak was observed in both mutant extracts with identical retention time and UV-vis spectra (Fig. 5). This peak was compared to a reference sample of cercosporin produced by wild-type *C. beticola*. The retention time of this peak was shorter than that of cercosporin suggesting a more polar metabolite. Comparison of the UV-vis spectra (Fig. 5*B-D*) of the unknown compound and cercosporin revealed nearly identical chromophores, suggesting close structural relation. The exact mass of the metabolite from the mutants was determined (Δ*842/CTB9*: m/z = 537.1762, Δ*843/CTB10*: m/z = 537.1757, [M+H+]), consistent with the elemental composition $C_{29}H_{28}O_{10}$. This mass is 2 Da greater than that of cercosporin (+2 hydrogens), which led to a proposed structure for pre-cercosporin (**2**) (Fig. 6). Alternative hydroquinones of cercosporin could be excluded simply on the basis of the UV-vis spectral information and chemical instability. The presence of a free phenol in pre-cercosporin in place of the unusual 7-membered methylenedioxy of cercosporin is consonant with the red shift of the long wavelength λ_{max} and the shorter HPLC retention time. To firmly support the tentative structure of pre-cercosporin, the crude extract of Δ*842*/*CTB9* was further purified by reverse-phase HPLC. To obtain sufficient material for ^{1}H-NMR analysis, extractions were performed quickly and in low light and reduced temperature to slow apparentpolymerization of pre-cercosporin. The relative instability of pre-cercosporin compared to cercosporin suggests a possible role for the methylenedioxy bridge in overall stability. Immediately evident in the ^{1}H-NMR spectrum (Suppl. Fig. S9A), apart from its

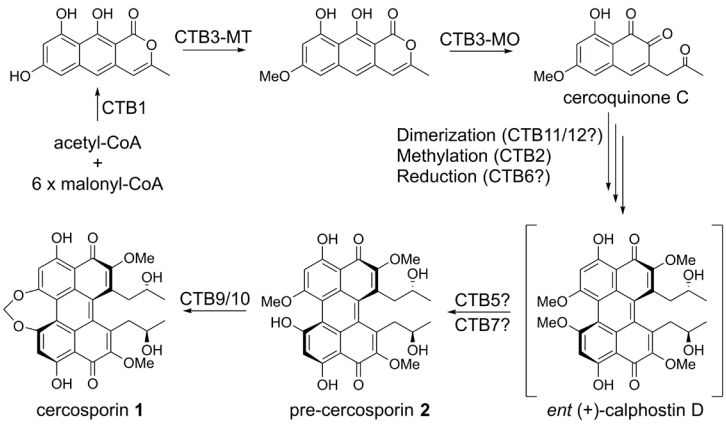

Figure 6. Proposed biogenesis of cercosporin. Tentative proposal for biosynthesis of cercosporin (1), incorporating newly discovered biosynthetic genes. Intermediates in brackets are logically inferred, and have not been directly observed. MT = methyltransferase, MO = monooxygenase.

overall similarity to that of cercosporin itself, was the absence of the methylenedioxy singlet at δ5.74 diagnostic of cercosporin, but the appearance of a new methoxyl signal at δ4.28 and a phenol at δ9.25. Consistent with the new asymmetry in pre-cercosporin, two strongly hydrogen-bonded *peri*-hydroxy groups could be seen far downfield at *ca.* 15 ppm and two aryl hydrogens were observed at δ6.92 and δ6.87. That these latter resonances are observed only in pairs, as are the two side chain methyl doublets at *ca.* 0.6 ppm, and the doubling of other signals imply that pre-cercosporin is formed as a single atropisomer having a helical configuration likely identical to that of cercosporin, although it is conceivable CTB9 or CTB10 sets the final stereochemistry.

^{13}C-NMR data were obtained by growing a larger number of PDA plates of Δ*842/CTB9* supplemented with 2 mM each [1-^{13}C]- and [2-^{13}C]-sodium acetate to equally enrich all polyketide-derived carbons (~3%/site). Working quickly to isolate and purify pre-cercosporin as above in low light and low temperature, both 1D and HSQC spectra of pre-cercosporin were acquired (Suppl. Fig. S9B and S9C). As seen in the ^{1}H-NMR spectrum, breaking the symmetry of cercosporin was evident in the observation of all 29 carbons in the ^{13}C-NMR spectrum, which notably revealed three methoxyl groups and diagnostic doubling of all resonances, save two overlapping pairs of signals. This behavior is fully in accord with the assigned structure of pre-cercosporin.

Identification of cercosporin from *Co. fioriniae*.

Since our initial phylogenomic analyses suggested that several *Colletotrichum* spp. harbored *CTB* clusters (Figs. 2, 3), we questioned whether the *CTB* cluster can be found in additional *Colletotrichum* spp. CTB protein orthology analysis revealed that eight out of the 13 *Colletotrichum* spp. hosted at Ensemble Fungi (https://fungi.ensembl.org/index.html) encode a similar set of CTB proteins as observed in *Co. higginsianum* (Suppl. Table S8). These eight species are plant pathogens of crops such as apple, safflower, melon, cucumber, and a variety of *Brassica* and cereal crops, as well as various tree species (41-47) (Suppl. Table S8, Figs. S10, S11). Remarkably, many species have lost several *CTB* genes such as the endophyte *Co. tofieldiae*, which has lost the cluster entirely (Suppl. Table S8, Figs. S10, S11).

Since earlier reports suggested the production of a "red pigment" by some *Colletotrichum* spp. such as the apple pathogen *Co. fioriniae* (48, 49), we questioned whether the red pigment was cercosporin. As a first step, two *Co. fioriniae* strains (HC89 and HC91) from our collection that were previously isolated from apple were assayed for cercosporin production using the KOH assay (50). No cercosporin-like pigment was observed in the medium under the same conditions that stimulate cercosporin production in *C. beticola*. Since epigenetic modifiers have been used to induce production of SMs in fungal species (51, 52), we considered whether this strategy could be used to induce cercosporin production in *Co. fioriniae*. Medium augmented with the histone deacetylase inhibitor trichostatin A (TSA) (51) induced production of a red cercosporin-like compound. To characterize this red metabolite, mycelia from both *Co. fioriniae* strains were extracted with ethyl acetate. Reverse-phase HPLC analysis of extracts of both strains revealed a peak with a retention time and UV-vis spectrum consistent with cercosporin in both extracts (Fig. 7*A*, *B*). The presence of cercosporin was confirmed by UPLC-ESI-MS (Fig. 7*C*). Moreover, complementary re-sequencing of both isolates on the Illumina platform followed by automated genome assembly and gene prediction confirmed the presence of a *CTB* cluster in both genomes (Suppl. Table S8, Fig. S11).

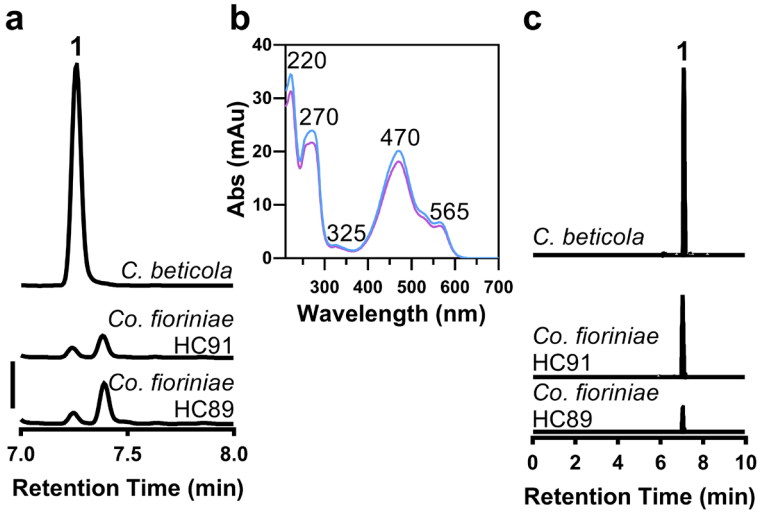

Figure 7. HPLC and UPLC-ESI-MS analysis of *Colletotrichum fioriniae* strains. **a)** HPLC chromatograms at 280 nm of wild-type *C. beticola* and *Co. fioriniae* HC89 and HC91. Scale bar indicates 100 mAu. **b)** UV-Vis spectra of cercosporin (7.25 min. retention time) extracted from *Co. fioriniae* HC89 (blue) and HC91 (purple). Wavelengths of relevant UV maxima are indicated. **c)** Extracted ion chromatograms (m/z = 535.1604) obtained by UPLC-ESI-MS, demonstrating cercosporin production in *C. beticola* and *Co. fioriniae* strains HC89 and HC91.

To assess whether *Co. fioriniae* produces cercosporin during apple infection, apples were inoculated with the pathogen, and tissue samples were collected every other day from one to 14 days post inoculation. Regardless of time-point or extraction methodology, we were unable to detect cercosporin from infected apple. However, by infiltrating apples with defined amounts of cercosporin, we determined the isolation efficiency is approximately 5% (Suppl. Fig. S12, S13). Consequently, any cercosporin produced by *Co. fioriniae* during infection is likely rapidly bound or modified by apple tissue such that its recovery by extraction is poor and below our level of detection by HPLC or ESI-MS. To provide additional support for the involvement of cercosporin in *Co. fioriniae* infection, we infiltrated cercosporin into apple fruit and visually compared the phenotype of the infiltrated area to *Co. fioriniae*-infected apple. At five days post infiltration/inoculation, cercosporin-infiltrated apple exhibited similar symptomology as *Co. fioriniae*-infected apple (Suppl. Fig. S14). We also quantified expression of *CTB1* using qRT-PCR since it is highly-expressed during cercosporin biosynthesis and is the first committed step in the pathway (21). In concordance with earlier results where no cercosporin was detected from cultures grown in vitro in the absence of TSA, we likewise did not detect *CTB1* expression from this source (Suppl. Fig. S15). However, *CTB1* was found to be induced ~11.9-fold in *in vitro* cultures with TSA and 2.5-fold in apple samples harvested 14 days post inoculation (Suppl. Fig. S15). Since amplification products were designed to span introns, amplicons were sequenced and verified to be derived from cDNA.

We also attempted to isolate cercosporin from *Co. higginsianum*-infected *Arabidopsis* leaves since previous studies indicated the distinct induction of this cluster from *in planta* appressoria (22 hours post inoculation; HPI) and the biotrophic phase of infection (40 HPI) but repressed during *in vitro* growth and the necrotrophic phase of infection (60 HPI) (44). Again, we were unable to detect cercosporin from infected *Arabidopsis* leaves regardless of the extraction methodology or time-point after inoculation. Given the relatively low amount of fungal biomass associated with the early time points when the *CTB* cluster is induced during *Arabidopsis* infection, we hypothesize that the amount of cercosporin produced *in planta* is below our current limits of detection.

Discussion

Several hypotheses exist for the maintenance of SM biosynthetic genes as clusters. In one, unlinked SM pathway genes are at a greater risk for dissociation during meiotic recombination (53) or chromosomal rearrangements (54). Additionally, clustering may facilitate strict coordination of gene expression, which may be particularly important during the biosynthesis of SMs that have potentially toxic or chemically unstable intermediates to ensure their efficient conversion to final end products (55). Horizontal transfer and maintenance of the *CTB* cluster specifically among plant pathogens suggests that it was critical for disease development in diverse pathosystems, including rice blast caused by *M. oryzae* and various anthracnose diseases caused by *Colletotrichum* spp. on many different crops. The *CTB* clusters in *Co. higginsianum* and *Co. graminicola* were reported as one of the few SM clusters between these species that are microsyntenic (44). Moreover, O'Connell et al. detected specific upregulation of the *CTB* cluster in *Co. higginsianum* during colonization of Arabidopsis (44). Indeed, nine of 14 *Co. higginsianum CTB* genes were among the top 100 most highly expressed genes *in planta*. Recent analysis of natural selection processes in *Co. graminicola* identified orthologs of *CTB* genes *CTB1* and *CFP* among the ~80 genes undergoing significant positive selection (56), further suggesting a role in pathogenicity. Interestingly, the *CTB* clusters of *Colletotrichum* spp. and *M. oryzae* contain additional genes; two short-chain dehydrogenases, an additional desaturase, a ferric-chelate reductase and an NmrA-like family protein, which has been reported (57) to act as negative transcriptional regulator.

The identification of cercosporin production in two isolates of *Co. fioriniae* may have significant implications for the apple packing, storage, and processing industries. Bitter rot, caused by *Colletotrichum* spp., is one of the top pre- and postharvest pathogens of apple (58). This disease is a major problem for the apple industry as it limits fresh fruit in the field and during storage, and has a quiescent stage allowing decay to occur on seemingly high-quality apples, only to come out of storage rotten (58, 59). Hence, contamination of processed apple products with cercosporin could be a significant health hazard. For example, other fungal-produced toxins (e.g. patulin, citrinin, penicillic acid) can contaminate processed apple products (60). Patulin, produced by *Penicillium* spp., is the most troubling as it is carcinogenic and consequently the United States and Europe have strict patulin limits in fruit juices and processed pome fruit products (60, 61). Future studies will focus on the role of cercosporin production during the *Colletotrichum*-apple fruit interactions in addition to assaying processed fruit products made

from apples with bitter rot symptoms to determine levels of the toxin in fruit. Although only *Co. fioriniae* and *Co. higginsianum* strains were analyzed for the ability to produce cercosporin, the identification of highly similar *CTB* clusters in other *Colletotrichum* species (Suppl. Table S8, Fig. S10, S11) suggest that cercosporin production may be wide-spread in this genus. Future studies directed towards analysis of *CTB* cluster expression among various *Co. fioriniae* isolates and apple cultivars will be necessary to confirm whether cercosporin is necessary for virulence of this pathogen.

The microsynteny outside of the established *CTB* cluster prompted us to test whether the flanking genes in *C. beticola* are also required for cercosporin biosynthesis. Notably, we observed that these flanking genes, similar to the established *CTB* genes, were up-regulated under cercosporin-inducing conditions. Furthermore, targeted gene replacement of *CTB9*, *CTB10*, *CbCTB11*, and *CTB12* completely abolished cercosporin biosynthesis, while replacement of *CTB9* and *CTB10* resulted in the accumulation of a new, red metabolite, defined here as pre-cercosporin. We thus conclude that the *CTB* cluster is significantly larger than previously described (19).

The isolation and characterization of a new intermediate in the cercosporin biosynthetic pathway, pre-cercosporin, strongly suggests that formation of the unique 7-membered methylenedioxy bridge in the final product is the result of a two-step process requiring three genes. First, one of two precursor aryl methoxyl groups of ent (+)-calphostin D (Fig. 6) is removed, followed by oxidative ring closure by CTB9, an apparent a-ketoglutarate-dependent dioxygenase, in collaboration with CTB10. The precise role of CTB10, a putative dehydratase, in ring closure is unclear, but it could serve to facilitate closure of the unfavorable 7-membered methylenedioxy ring. In contrast, a single cytochrome P450 is known to convert two aryl *ortho*-methoxyl groups into the relatively more common 5-membered methylenedioxy group in alkaloid biosynthesis (62). We attribute the single demethylation to an oxidative process possibly carried out by the flavin-dependent enzymes CTB5 or CTB7. CTB6 correlates to the SDR NAD(P)H-binding superfamily of oxidoreductases and could install the side chain hydroxyl groups stereospecifically. Owing to the extreme instability of most pathway intermediates and the role feedback inhibition in response to these metabolites could play, our experience dictates that analysis of pathway knockouts alone will not lead to the full determination of cercosporin biosynthesis. Biochemical evaluation of the individual enzymes, as has been done with CTB3 (21), with synthetic substrates and product standards will be necessary to accomplish this task.

A tentative cercosporin biosynthesis scheme was recently proposed (21) without knowledge of the expanded *CTB* cluster. However, in light of the identification of pre-cercosporin and the potential functions of the other newly discovered *CTB* genes, the previously proposed biosynthetic pathway (21) will have to be revised. While these investigations will be reported in due course, we suspect the newly discovered fasciclin/laccase pair (CTB11/12) may act early in the pathway to dimerize the product of CTB3 (21) to the first perylenequinone intermediate, which would have precedent in synthetic chemistry (63) and in simpler laccase-mediated aryl–aryl dimerizations (36, 64, 65) (Fig. 6). CTB1 is an iterative, non-reducing polyketide synthase whose product is *nor*-toralactone (20). CTB3 is a bifunctional enzyme, *O*-methyltransferase

and FAD-dependent monooxygenase, that carries out sequential *O*-methylation in the presence of *S*-adenosylmethionine (SAM) and oxidative decarboxylation to cercoquinone C (21). We hypothesize intermediate steps of *O*-dimethylation and side chain ketone reduction, in unspecified order, are mediated by CTB2 and potentially CTB6, respectively, as noted above.

Despite sustained research on cercosporin for several decades, there are significant knowledge gaps in cercosporin biosynthesis. Our data shed new light on cercosporin biology that will have significant impact on cercosporin research specifically and perylenequinone research in general. The finding that at least one species in the important plant pathogenic genus *Colletotrichum* can produce cercosporin has significant implications for disease management. Moreover, since *Co. fioriniae* may secrete cercosporin into apple food products that may be directly consumed by humans, the toxic effects of cercosporin on human health may need to be considered.

Materials and Methods

For further information, see *SI Appendix* Materials and Methods and figshare under DOI: 10.6084/m9.figshare.4056522. Custom code is permanently archived at Zenodo under DOI: 10.5281/zenodo.1156551.

Fungal genomic DNA was isolated from mycelia scraped from the surface of agar Petri plates. Library preparations and sequencing on the Illumina platform was performed by BGI Americas Corporation. For *C. beticola*, three genomic libraries with increasing insert size (500 bp, 5 Kbp, 10 Kbp) were sequenced. For *C. berteroae*, *C. nicotianae*, and for *Co. fioriniae* strains HC89 and HC91, single, short insert libraries (500 bp) were sequenced. For *C. beticola* specifically, optical maps were prepared using the Argus (OpGen) and BioNano Genomics platforms and subsequently used to scaffold contigs into large supercontigs. A combination of ab initio gene prediction, homologous protein alignment, and transcript alignment followed by extensive manual curation was used to prepare draft gene models for *C. beticola*. *C. beticola* trained Augustus parameters were used for automated protein-coding gene modelling in the case of *C. berteroae*, *C. canescens* and *C. nicotianae*. Genome assemblies and annotations, if applicable, are deposited at NCBI GenBank and listed under BioProject PRJA270309. Accession numbers for *C. beticola*, *C. berteroae*, *C. nicotianae* and *Co. fioriniae* strains HC89 and HC91 are: LKMD00000000, PNEN00000000, POSS00000000, and PNFH00000000 and PNFI00000000, respectively.

Mycelial plugs of wild-type and mutant *C. beticola* were placed on top of eight "thin" potato dextrose agar (PDA, Difco) plates (3.0 mL PDA per 50 mm Petri plate). Cultures were incubated at 22 °C for one week under continuous light. PDA and mycelia were ground under liquid nitrogen and lyophilized to dryness twice. The resulting powder was resuspended in water acidified with HCl (pH <1), allowed to sit 10 minutes, and filtered. The filtrate was extracted thrice with ethyl acetate. These extracts were pooled washed with brine, and evaporated to dryness. The extracted metabolites were resuspended in 500 µL methanol and analyzed by HPLC on an Agilent 1200 fitted with a Kinetex XB-C18 column (4.6 x 75 mm, 2.6 µ, Phenomenex). Injections

of 1 µL were run at 1.25 mL/min with a linear gradient of 5% A/95% B to 95% A/5% B over 10.8 min, where solvent A was acetonitrile + 0.1% formic acid and solvent B was 0.1% formic acid. Chromatograms were monitored at 436, 280, and 210 nm, and UV-vis spectra were recorded over a range of 210-800 nm. High resolution mass data were obtained from a Waters Acquity/ Xevo-G2 UPLC-ESI-MS in positive ion mode.

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Supporting material for this chapter can be accessed under https://doi.org/10.1073/pnas.1712798115

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Chapter 6

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

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Abstract

Perylenequinones are a family of structurally related polyketide fungal toxins with nearly universal toxicity. These photosensitizing compounds absorb light energy which enable them to generate reactive oxygen species that damage host cells. This potent mechanism serves as an effective weapon for plant pathogens in disease 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 (1-5). For example, in some occasions SMs are involved in symbiotic relationships where microbial symbionts provide an antibiotic armory against secondary infection to the symbiotically colonized plant in return for nutrients and protection (5). A major class of fungal SMs are the polyketides (6). For the biosynthesis of fungal aromatic polyketides, non-reducing polyketide synthases (NR-PKSs) play a central role as mediators of the first biosynthetic step (6-9). Such PKS genes contain multiple domains that work conjointly, of which the β-ketoacyl synthase (KS), acyltransferase (AT), and acyl-carrier protein (ACP) domain are indispensable (6, 7, 9, 10). By using the domains iteratively, a PKS generates a metabolite backbone which can be modified by other enzymes to yield the final metabolite (6, 9, 11). The genes encoding these decorating enzymes are often found in direct proximity to the PKS gene to form a biosynthetic gene cluster (BGC) pathway (6, 12). In addition, BGCs contain regulatory elements and transporters involved in shuttling the final secondary metabolite from the cell, and in the case of toxic metabolites, genes encoding auto-resistance proteins (4, 13).

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 (13-15). The ubiquitous presence of the *CTB* gene cluster in the genus is likely explained by its role as a virulence facilitator (15-17). Recently, de Jonge et al. (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 (9, 11, 13). The majority of assessed species from the genus *Colletotrichum*, a large genus of crop and/or ornamental plant pathogens (18), were shown to harbor full- to partial-length *CTB* gene clusters, of which the post-harvest apple fruit pathogen *Co. fioriniae* was shown to produce cercosporin (13). The core gene of the *Cercospora CTB* gene cluster is the NR-PKS gene *CTB1* (19), which is flanked at both sides by nine genes that putatively encode decorating enzymes (CTB2, CTB3, CTB5, CTB6, CTB7, CTB9, CTB10, CTB11 and CTB12) (13). Besides those ten 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 auto-resistance (13, 20, 21). Upon activation, all *CTB* pathway enzymes work in a well-orchestrated manner to synthesize the metabolite from backbone formation to secretion of the toxin into the environment whilst 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 (16, 22-25). Exposure to visible and near-UV light energetically activates perylenequinones to an excited triplet state that reacts with oxygen to form reactive oxygen species (25, 26). This photodynamic activity can be attributed to the 3,10-dihydroxy-4,9-perylenequinone chromophore backbone that is shared among

perylenequinones (27). Structural differences between perylenequinone family members are mostly due to divergent side chains attached to the mutual backbone structure (28) (Fig. 1). For example, the methylenedioxy bridge is a unique feature of cercosporin and is absent in other perylenequinones such as hypocrellin, elsinochrome and phleichrome (Fig. 1) (13, 29).

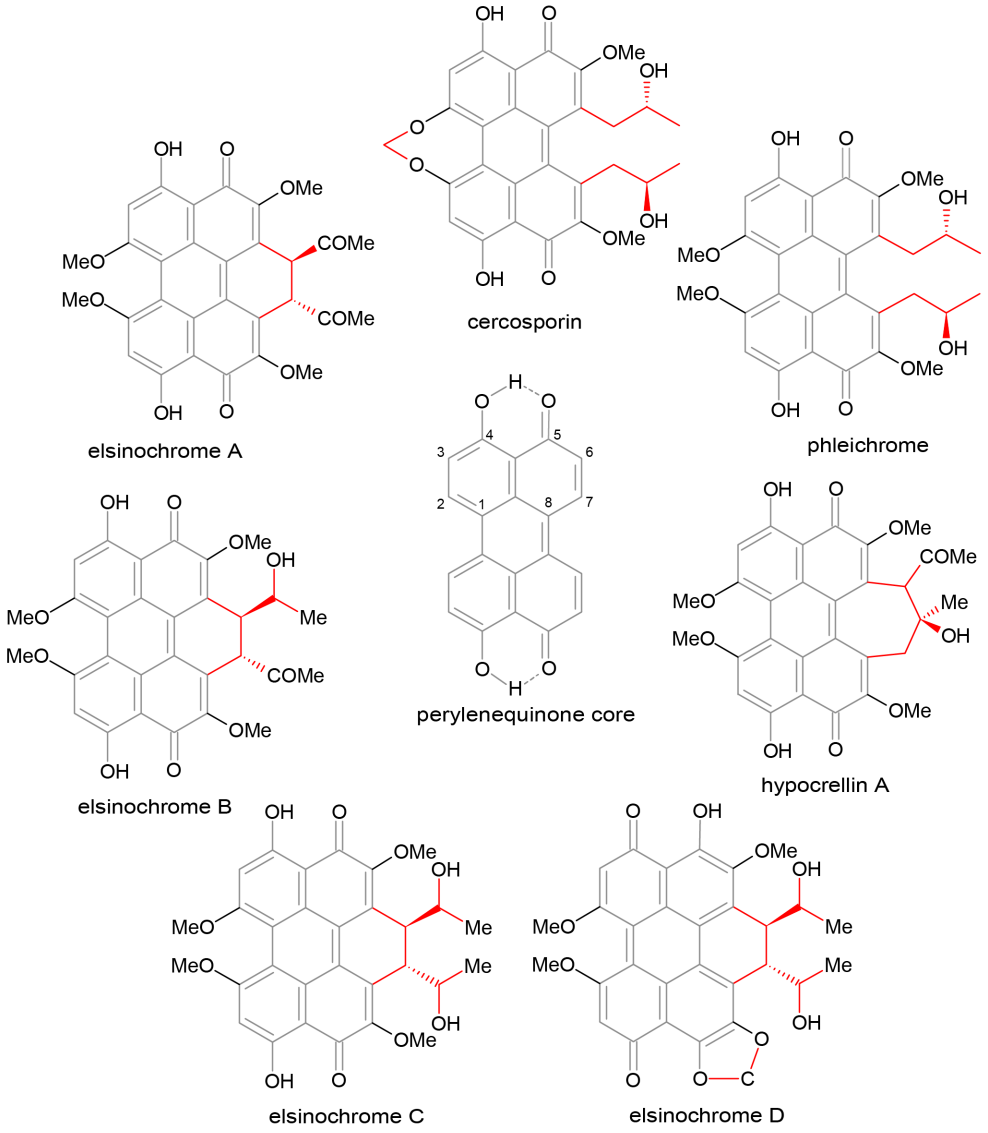

Figure 1. Structures of related perylenequinones. Cercosporin synthesized by *Cercospora* spp., phleichrome by *C. phlei* and elsinochromes A, B, C, and D produced by *E. fawcettii* are structurally related as they share a common perylenequinones backbone (center; indicated in the molecule structure in gray). Structural differences between the molecules are mostly due to various side chains (indicated in red). Differences between perylenequinones are observed at positions 2, 2'and 7, 7'.

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 *Shiraia bambusicola* gave compelling evidence that *SbaPKS* encodes the PKS orchestrating hypocrellin biosynthesis (30, 31). Similarly, targeted disruption of *EfPKS1* in the citrus scab pathogen *Elsinoë fawcettii* appeared to abrogate elsinochrome production (32). Likewise, Cppks1 was suggested to be responsible for PKS activity for phleichrome production in the purple eyespot pathogen *Cladosporium phlei* (33). However, we have previously used KS domain phylogeny to associate PKS genes with the final perylenequinone product (13). During the course of these analyses, we identified PKS genes for *E. fawcettii* and *C. phlei* that were not previously attributed to these perylenequinones, which prompted us to re-evaluate the findings of Liao *et al*. (2008) (32) and So et al. (2015) (33). Interestingly, Liao et al. (2008) (32) also carried out phylogenetic analysis of EfPKS1 with other PKSs which indicated that EfPKS1 clustered closely to a diverse set of fungal non-reducing PKSs involved in biosynthesis of the toxins cercosporin, aflatoxin and sirodesmin, but also with PKSs involved in pigment production such as dihydroxynaphthalene (DHN)-melanin. 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 (34-36). In *Mycosphaerella fijiensis*, 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 (37).

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-

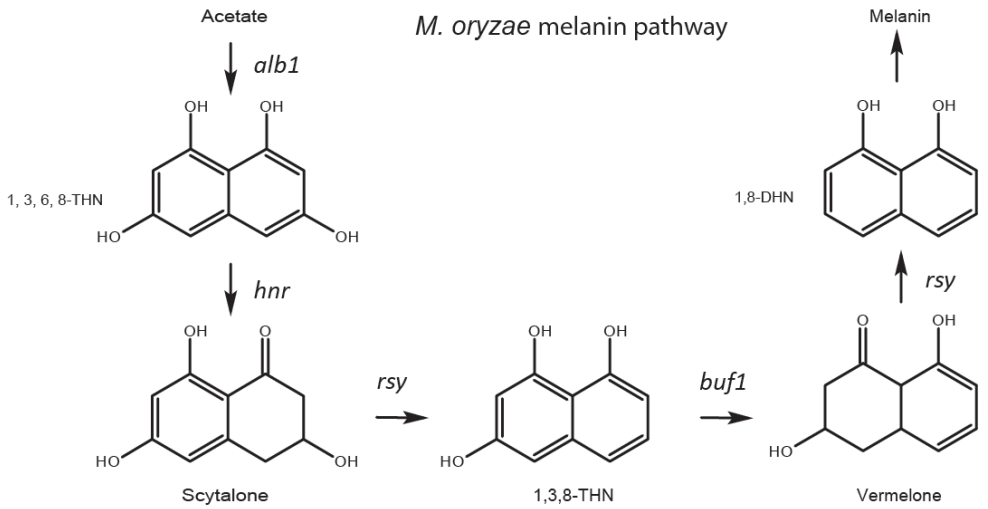

Figure 2. Schematic DHN-melanin biosynthesis pathway of *M. oryzae*. In the first biosynthetic step, the PKS ALB1 forms 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN or T4HN) by ketide cyclization. Reduction by the tetrahydroxynaphthalene reductase 4HNR results in the formation of scytalone which will be dehydrated by RYS1, a scytalone dehydratase, to yield trihydroxynaphthalene (T3HN). The T3HN reductase BUF1 subsequently reduces T3HN to vermelone followed by a dehydration step mediated by RYS1 to form dihydroxynaphthalene (2HN), the immediate precursor of melanin.

melanin production is known to be mediated by a four-gene cluster which is regulated in hyphae by the transcription factor Pig1 (Fig. 2) (38-41). 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 1") 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 (42, 43). Two alternative routes can be found in the necrotrophic gray mold fungus *B. cinerea*. In this case, the 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 (44). 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-trihydroxynapthalene (1,3,8-THN or T3HN). Subsequent reduction by a HN reductase yields vermelone which is subsequently dehydrated to form 1,8-DHN; an immediate precursor of melanin (38-40, 45).

In this manuscript, we show that the gene clusters housing *Cppks1* and *EfPKS1* 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. Due to its detailed characterization, the established *M. oryzae* melanin cluster was used as reference in our alignments of putative DHN-melanin gene clusters of *C. beticola*, *E. fawcettii*, *C. phlei*, and *S. bambusicola* to illustrate the high level of homology among the DHN-melanin BGCs. Consequently, we also sought to establish the BGCs involved in production of elsinochrome in *E. fawcettii* and phleichrome in *C. phlei*, and included 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

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 platform (paired-end, 100-bp reads). Raw reads were processed and assembled by SPAdes (version 3.9.0) 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 numbers for these assemblies are 13 and 676 Kb, and 44 and 238 Kb. Following genome assembly, we used Augustus (version 3.2.1) with default settings (46) and the previously devised *C. beticola* training parameters (13) to predict 9,519 (mean length 1,675 bp and ~2.5 exons/gene) and 11,316 (mean length 1,624 bp and ~2.3 exons/gene) protein-coding genes for *E. fawcettii* and *C. phlei* respectively. Finally, protein function as well as putative localization was predicted by Interpro (47) scanning and yielded annotations for 9,253 out of 9,519 *E. fawcettii* proteins and 10,870 out of 11,316 *C. phlei* proteins. Considering only hits to Pfam, SMART, CDD or SUPERFAMILY databases, 7,450 (78%) and 8,479 (75%) genes were annotated for *E. fawcettii* and *C. phlei*, respectively.

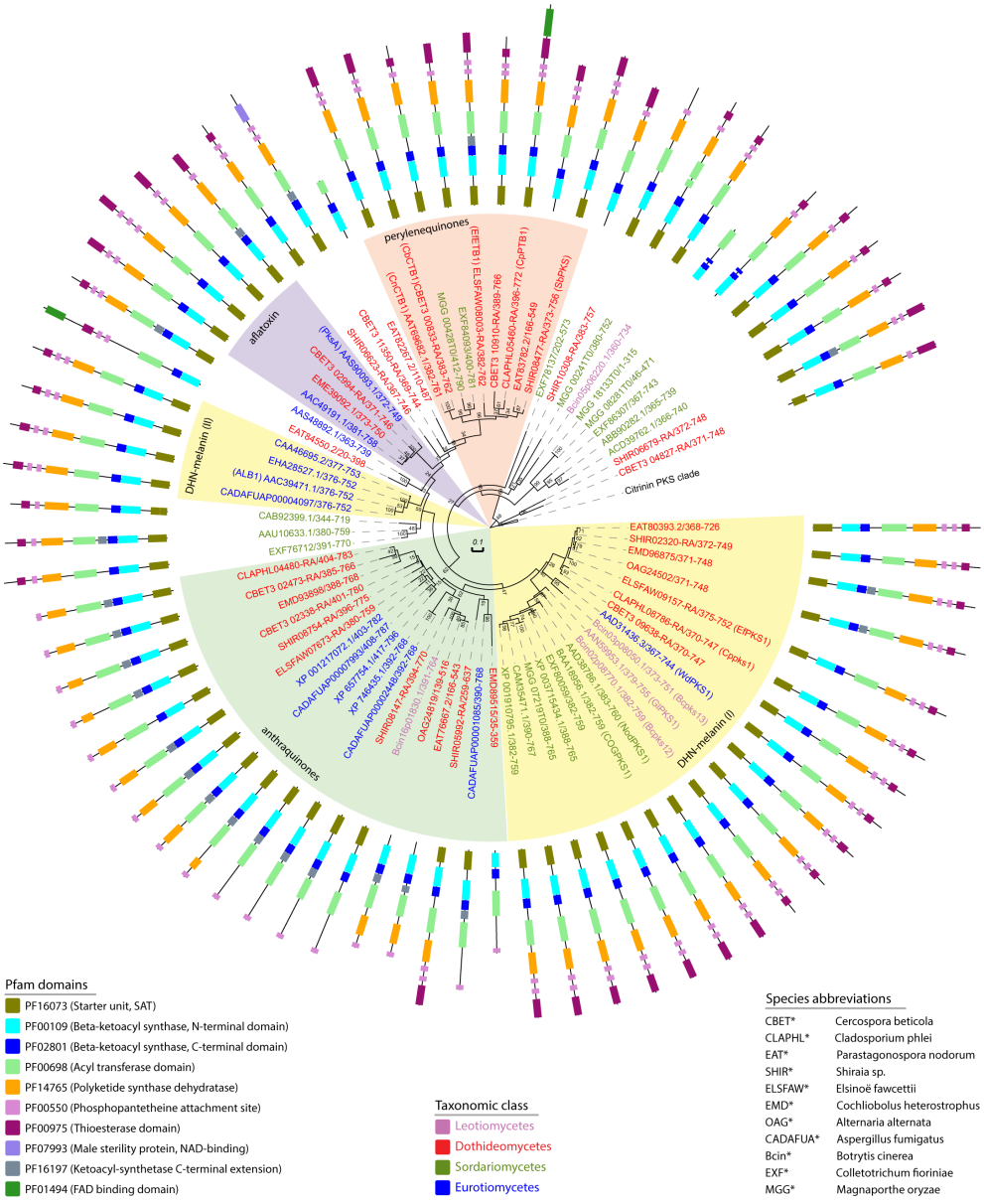

Figure 3. Phylogeny of PKS genes of related Ascomycetes revealing distinct DHN-melanin and perylenequinone subclades. Circular maximum likelihood phylogenetic tree illustrating the phylogenetic relationship of all predicted non-reducing polyketide synthase (PKS) from the selected species set (Suppl. Table 1) plus those derived from the set of PKSs used by Collemare *et al.* (2014) (48). The tree was constructed by maximum-likelihood analysis of aligned full-length β-ketoacyl synthase domains. The outside ring indicates domain architecture of each PKS determined by Pfam domain annotation. Protein accessions are colored depending on the taxonomic class of the producing species. The corresponding species identity for each protein can be found in the bottom left corner. Established biosynthetic end products for a subset of the listed PKSs is indicated by the background color, highlighting two DHN-melanin sub-groups, naphthoquinones, anthraquinones, perylenequinones, aflatoxin-like compounds and resorcylic acid lactones.

PKS genealogy and prediction of function

To study the level of conservation of PKSs and associated pathways involved in the biosynthesis of different perylenequinones, we mined the genomes of both perylenequinone producers and non-producers for non-reducing PKSs (Suppl. Table 1). Subsequently, the phylogenetic relationships between these PKSs and those of previously characterized PKSs from selected species as found in Collemare *et al*. (2014) (48) (Suppl. Table 1) were determined by aligning the highly conserved β-ketoacyl synthase (KS) domains of each PKS (Fig. 3). This genealogy revealed distinctive clade formation where PKSs with confirmed involvement in biosynthesis of structurally similar metabolites were observed to cluster. The clades were categorized as perylenequinone, aflatoxin, anthraquinone, or DHN-melanin biosynthesis depending on the function of confirmed PKSs they harbored (Fig. 3). Interestingly, the PKSs EfPKS1 (32)/ [ELSFAW09157-RA (this study)] from *E. fawcettii* and Cppks1 (33)/ [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, EfPKS1 and Cppks1 formed a clade with confirmed melanin PKSs, including Bcpks12 and Bcpks13 of the gray mold fungus *B. cinerea* (44), Wdpks1 of the zoopathogenic black yeast *Wangiella* (*Exophiala*) *dermatitidis* (49), GlPKS1 of the filamentous fungus *Glarea lozoyensis* (50), NodPKS1 of an endophytic *Nodulisporium* strain (51), and COGPKS1 of the cucumber anthracnose causal agent *Co. lagenarium* (52) and 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 with extensive collinearity to established DHN-melanin clusters (Fig. 3) suggests a role in melanin production and hints that *EfPKS1* and *Cppks1* were previously misannotated as perylenequinone biosynthesis genes (32, 33).

The cercosporin PKSs in *C. beticola* (CbCTB1), *C. nicotianae* (CnCTB1), and *Co. fioriniae* (EXF84093) form a perylenequinone clade with the previously confirmed hypocrellin PKS (SbaPKS) (30, 31), ELSFAW08003 from *E. fawcettii*, CLAPHL05460 from *C. phlei* as well as with the putative perylenequinone PKSs in *P. nodorum* (EAT83782.2), *M. oryzae* (MGG_00428), and the *C. beticola* CbCTB1 paralog CBET3_10910 (Fig. 3). As phylogenetic conservation can be an indication of related metabolite production (13), this clustering suggests that PKSs of this clade are involved in biosynthesis of the perylenequinones. Therefore, we suggest renaming *ELSFAW08003* to *EfETB1* for elsinochrome toxin biosynthesis gene 1, and *C. phlei CLAPHL05460* to *CpPTB1* for phleichrome toxin biosynthesis gene 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, synteny of the predicted BGCs of orthologous PKS genes was assessed. Using the established *C. beticola CTB* gene cluster and *S. bambusicola* hypocrellin gene cluster as references, putative perylenequinone orthologous gene clusters in *E. fawcettii*, *C. phlei*, *P. nodorum*, *M. oryzae*, and *Co. fioriniae* were aligned (Fig. 4A). Although there is evidence for gene loss and gain between the perylenequinone BGC alignments, multiple core genes are shared between cercosporin, hypocrellin, and the predicted BGCs for

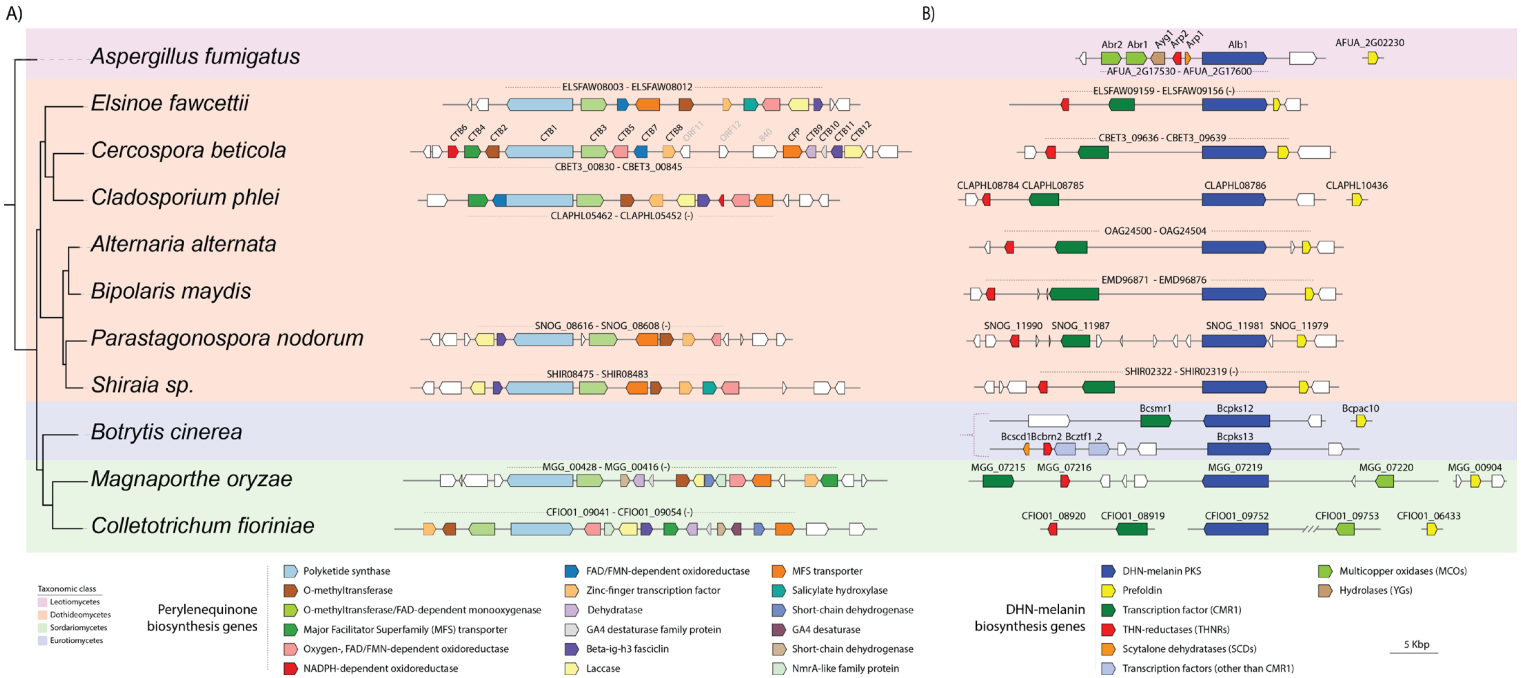

Figure 4. Synteny and rearrangements of conserved perylenequinone and DHN-melanin BGCs. Phylogenetic tree of Ascomycetes used in this study based on mash protein-level kmer hash overlaps. Alignments were based on MultiGeneBlast with a selected set of genomes and an input BGC as query. Alignment of established and putative perylenequinone BGCs of E. fawcettii, C. beticola, C. phlei, P. nodorum, S. bambusicola, M. oryzae, and Co. fioriniae (A). For the DHN-melanin BGC alignment, putative and established DHN-melanin BGCs of E. fawcettii, C. beticola, C. phlei, P. nodorum, S. bambusicola, M. oryzae, Co. fioriniae, A. fumigatus, A. alternata, B. maydis (C. heterostrophus), and B. cinerea were aligned (B). 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 colored relative to the C. beticola CTB cluster genes while DHN-melanin BGC genes are color coded relative to M. oryzae DHN-melanin. Color key and annotated functions are explained in the legend below the cluster graphics.

elsinochrome and phleichrome (Fig. 4A). Overall, eight genes are shared between the cercosporin, hypocrellin and predicted elsinochrome and phleichrome BGCs (Figs. 4A, 5). When compared to these perylenequinone pathways, the *CTB* gene cluster has two additional genes; a putative α-ketoglutarate-dependent dioxygenase (*CTB9*) and a candidate dehydratase (*CTB10*) that have been shown to be involved in the formation of the methylenedioxy bridge (13). 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. 4A). *ELSFAW08009* and *SHIR08482* are annotated as 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 homologs *CTB4*, *CTB6*, and *CTB7* compared to the cercosporin pathway (Fig. 4A).

Similarly, predicted DHN-melanin clusters of *C. beticola*, *C. phlei*, *E. fawcettii*, *S. bambusicola sp. slf14*, and *Co. fioriniae* 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* (Figure 4B). All BGCs share homologous PKS genes, a THN-reductase, and a prefoldin-encoding gene. Prefoldins 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. fioriniae* contain a transcription factor with homology to *M. oryzae* Pig1 and *Co. lagenarium* CMR1, which are often observed in other established melanin clusters (40).

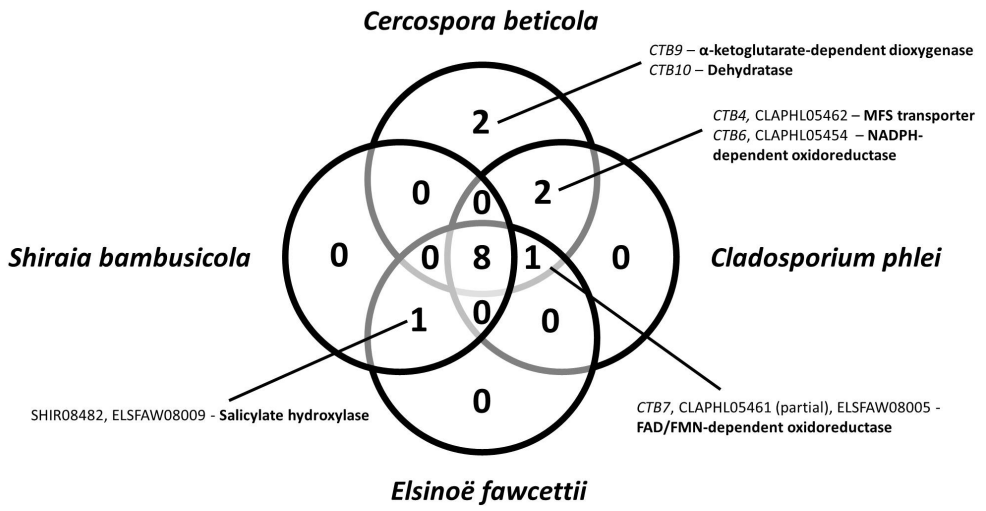

Figure 5. Conserved and unique genes in the confirmed or predicted perylenequinone BGCs of *C. beticola*, *E. fawcettii*, *C. phlei*, and *S. bambusicola*. Venn diagram highlights the number of shared BGC genes of the cercosporin, elsinochrome, phleichrome, and hypocrellin pathways.

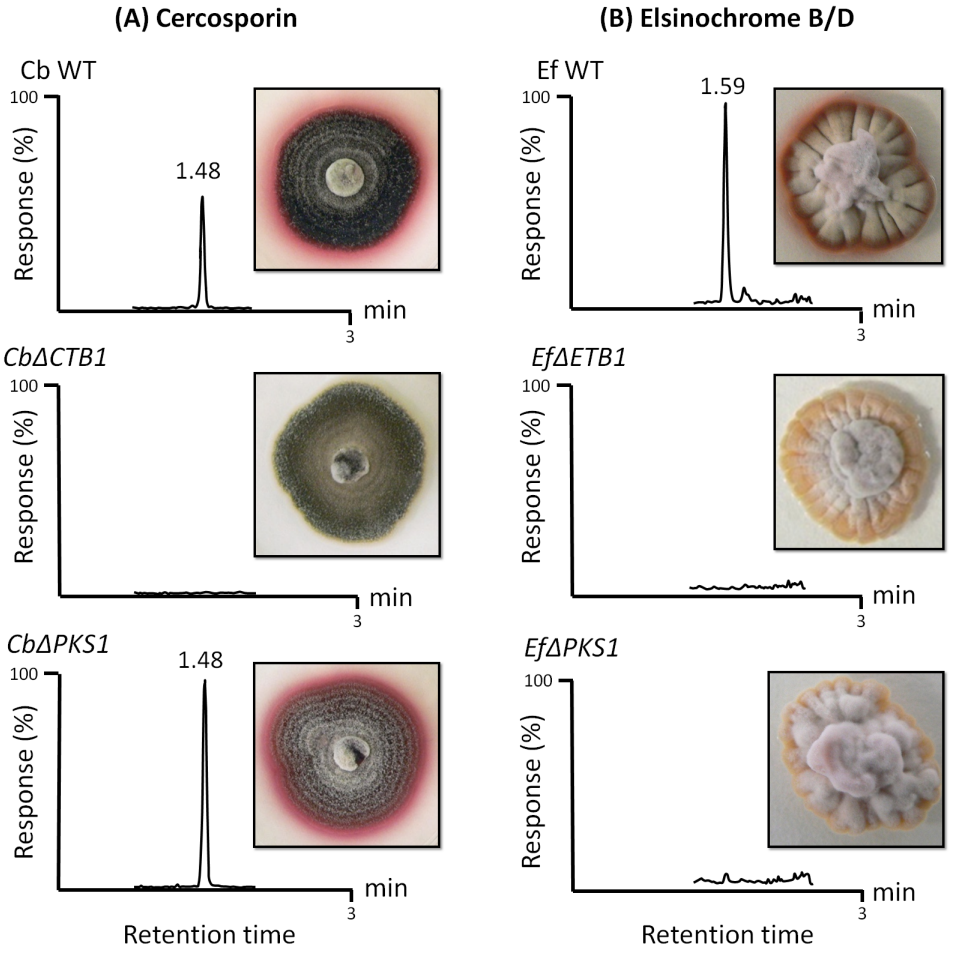

Figure 6. Perylenequinone toxin detection in *C. beticola* and *E. fawcettii* perylenequinone and melanin PKS mutants compared to wild type strains after growth under perylenequinone-inducing conditions. Representative UPLC mass-selective detection of cercosporin (column A) and elsinochrome B/D (column B) are shown for each fungal strain (minimum of 2 plate extracts per strain). Cercosporin (column A) was present in *C. beticola* wild type and the *CbΔPKS1* mutants at a (retention time 1.48 min) but not in the *CbΔCTB1* mutants (the cercosporin standard produced a mass-selective chromatogram with an identical retention time; data not shown). An elsinochrome B/D peak (column B) was present only in wild type *E. fawcettii* strains, retention time 1.59 min, and was undetectable in both *EfΔETB1* and *EfΔPKS1* mutants (no chemical-grade standard available).

Targeted replacement and characterization of perylenequinone and melanin PKS genes

The predicted perylenequinone and melanin PKS genes for *C. beticola* and *E. fawcettii* 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 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. 6). For *C. beticola*, the production of cercosporin was abrogated in Δ*CbCTB1* mutants but Δ*CbPKS1* mutants were still able to produce cercosporin (Fig. 6A). There were no obvious differences in growth rate for either of the *C. beticola* mutants versus the wild type strain. Additionally, Δ*CbPKS1* mutants had a pale buff color as opposed to the dark grey pigmentation observed in wild type strains (Fig. 6A). For *E. fawcettii*, both the Δ*EfETB1* and Δ*EfPKS1* mutant strains lacked elsinochrome production whilst the toxin was present as a deep red pigment in the wild type (Fig. 6B). The amount of melanin present in the cultures was determined spectrophotometrically, showing that Δ*PKS1* and Δ*CTB1*/Δ*ETB1* mutants for both species had a significantly lower melanin content than their respective wild types ($P < 0.05$) (Fig. 7A and B). The melanin content of ectopic mutants in both *C. beticola* and *E. fawcettii* did not significantly differ from their wild type ($P > 0.05$), demonstrating that the decreased melanin content observed for Δ*PKS1*, Δ*CTB1* and Δ*ETB1* mutants was unlikely an effect of protoplast transformation and was instead caused by the disruption of the targeted gene. The *C. beticola* and *E. fawcettii* Δ*PKS1* mutants unexpectedly exhibited some melanin extract absorbance at 475 nm, which is likely due to background absorbance.

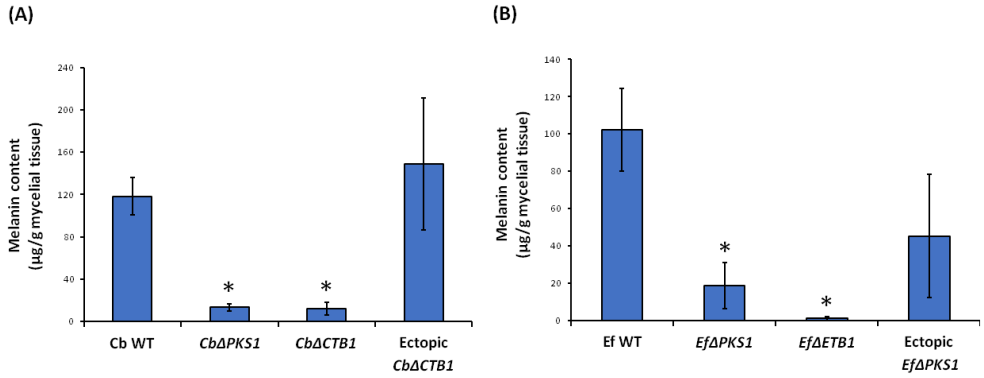

Figure 7. Melanin production in *C. beticola* and *E. fawcettii* perylenequinone and melanin PKS mutants compared to wild type strains. The mean melanin content of three individual fungal cultures (µg melanin/g of mycelial tissue ± standard error) in (A) *C. beticola* wild type (WT), melanin mutants (*CbΔPKS1*), cercosporin mutants (*CbΔCTB1*) and (B) *E. fawcettii* WT, melanin mutants (*EfΔPKS1*), and elsinochrome mutants (*EfΔETB1*). Ectopic mutants were included as positive controls. Significant differences ($P< 0.05$) indicated by *.

Discussion

Phylogenetic analysis based on PKS KS domain conservation can help to predict SM structure and gene evolution (6, 7). 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 PKS genes involved with perylenequinone biosynthesis including CbCTB1, the well-studied *Cercospora beticola* PKS essential for cercosporin biosynthesis, and the PKS gene of the hypocrellin pathway in *S. bambusicola sp. slf14*. We also observed clustering of PKS genes involved in DHN-melanin formation such as Bcpks12 and Bcpks13 of *B. cinerea* and COGPKS1 of *Co. lagenarium* (Fig. 3). As previously reported (32), 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 *et al*. (2015) (33) to hypothesize that Cppks1 was involved in phleichrome production. Our KS domain alignment confirms the phylogenetic analysis by Liao *et al*. (2008) (32) where EfPKS1 and Cppks1 form a cluster with established DHN melanin biosynthesis PKSs of other Ascomycetes (Fig. 3). Consequently, we used comparisons to well-characterized melanin BGCs in various Ascomycetes to show that PKS genes 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. 4B). Besides PKS phylogeny, 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 Δ*EfPKS1* and Δ*CbPKS1* displayed pale phenotypes characteristic to previously described melanin-deficient mutant strains (53) (Fig. 6A and B) and had reduced melanin production (Fig. 7A and B).

Interestingly, the *C. beticola* Δ*CbPKS1* mutant was still able to produce cercosporin, while no elsinochrome production was detected in the *E. fawcettii* Δ*EfPKS1* mutant. Since elsinochrome-deficiency in *E. fawcettii* Δ*EfPKS1* mutants was also reported by Liao *et al*. (2008) (32), we suspect that the phenotype observed is a pleiotropic effect. For example, pleiotropic effects of melanin were demonstrated in *Rynchosporium commune*, where the degree of melanization was positively correlated with both virulence and fungicide resistance (54). It is tempting to speculate that the knock-on effect of melanin production on levels of other secondary metabolites could influence these phenotypes. Toxin and melanin biosynthesis pathways have also been suggested to overlap in *Curvularia lunata*, where the higher virulence of one strain compared to another was associated with expression differences in both toxin and melanin biosynthesis pathways (55). The reduction in virulence observed for their *EfPKS1* deletion mutant (32) is not surprising since melanin has been reported to be a virulence factor for many filamentous fungi (34-36). 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 (56, 57). 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 (58). 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 suggest that the *EfPKS1* and *Cppks1* genes that were formerly predicted to be involved with elsinochrome and phleichrome biosynthesis were likely incorrectly annotated in previous publications and are involved in DHN-melanin biosynthesis.

To identify the legitimate elsinochrome and phleichrome 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*, which hinted at their contribution to perylenequinone biosynthesis (Fig. 3). In line with these initial functional predictions, alignments of the corresponding predicted gene clusters display high similarity and gene conservation within each clade (Fig. 4A). Also, structural differences between perylenequinones can be explained by comparing the predicted metabolite clusters on a gene level. For example, cercosporin and phleichrome only differ in the additional methylenedioxy bridge that is found in the cercosporin molecule (Fig. 1). Accordingly, the predicted phleichrome biosynthesis pathway lacks *CTB9* and *CTB10* that have been shown to be responsible for methylenedioxy bridge formation (13). Site-directed gene replacement of *EfETB1* 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. 6A and B). Since SM production relies on different environmental conditions, not every medium is suitable to activate SM production (59, 60). For *C. beticola*, research on cercosporin-inducing conditions resulted in the identification of thin PDA plates in combination with natural light as the induction condition of choice (61-63), which was shown here to stimulate elsinochrome production. Interestingly, we also observed a pleiotropic effect of perylenequinone biosynthesis on melanin content, with the toxin PKS mutants exhibiting reduced melanin levels compared to wild type (Fig. 7A and B).

In conclusion, we have shown that it is possible to identify 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 single out 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*, *P. nodorum*, 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.

Materials and Methods

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 (58). Library preparation (500 bp) and subsequent paired-end (PE) genome sequencing was done by BGI via the Illumina platform. 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 (13) in Augustus (version 3.2.1). Genome sequences and annotations are submitted to NCBI 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 *et al*. (2018) (13). In short, we used Pfam domain scanning analyses by HMMER3 (64) 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* (Slf14), *P. nodorum* (SN15), *C. heterostrophus* (C5), *A. alternata* (SRC1lrK2f), *A. fumigatus* (Af293), *B. cinerea* (B05.10), *Co. fioriniae* (PJ7), and *M. oryzae* (70-15) that were obtained from NCBI GenBank or Ensemble Fungi. In total we identified 240 proteins across these 11 proteomes. In addition, we added 70 PKSs from Collemare *et al*. (2014) (48) and Cppks1 (AFP89389.1) from So *et al*. (2015) (33). All selected proteins for further analyses are listed in Supplementary Table 1. All 311 PKS proteins were subsequently aligned by Mafft (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 non-reducing PKSs for further analysis, as defined previously by Kroken *et al*. (2003) (10). The final phylogenetic tree and figure was prepared in EvolView (65). In this tree, we collapsed the outgroup clade with 20 members containing PKSs involved with citrinin biosynthesis, as indicated in Figure 4. Inclusion in the final set of 74 non-collapsed, non-reducing PKSs is indicated in Supplementary 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 beforementioned proteomes using orthoFinder (66). Subsequently, we used the MultiGeneBlast algorithm (multigeneblast.sourceforge.net), integral part of antiSMASH (67), to prepare gene-by-gene cluster alignments across all species and we then re-colored individual genes within each gene cluster according to the protein family analysis.

Deletion mutant generation

Site-directed gene replacements of *CTB1* and *CbPKS1* in *C. beticola* strain 1-90 and of *EfETB1* and *EfPKS1* in *E. fawcettii* strain CBS 139.25 were generated using the split-marker approach as described in Bolton *et al*., 2016. Primers are listed in Suppl. 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. Additionally, absence of an amplicon using target gene- specific primers reconfirmed deletion of the target gene (Suppl. 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, DifcoTM, BD Diagnostic Systems, Sparks, USA) plates (3.0 mL PDA in a 50 mm Petri plate, amended with 150 µg ml-1 hygromycin B (Roche, Mannheim, Germany) (for mutant strains) under a natural light-dark cycle at 21 °C. C. beticola was grown for 7 days and E. fawcettii for 14 days before toxin extraction.

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). 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, USA) and 21, 500 as the molar extinction coefficient (68). Extracts were diluted to ~100 pg µl-1 with methanol and centrifuged at 3,000 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, USA) 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 0.16mL/min.

Cercosporin and elsinochrome (isomers B and D) were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) using a Waters (Milford, MA; USA) 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 x 30 mm (1.7 µm) Acquity CSH C18 column protected by a 2.1 x 5 mm CHS guard column (Waters; Milford, MA, USA). 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 minutes, 5% A at 2.1 minutes, and held at 5% A until 2.5 min when solvent A was ramped back to 95% A at 3 minutes. Solvent composition was held constant until the end of the run time at 4 min. The column temperature was 30°C.

Melanin production assay

Total mycelial tissue from each of wild type and mutant *C. beticola* and *E. fawcettii* strains was excised from 14 day old cultures (mycelial plugs grown on full strength PDA at 21°C with a natural light-dark cycle) and weighed before extracting melanin according to Gadd (1982) (69). 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 mins, 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 2M sodium hydroxide at 50 °C. A spectrophotometric assay was used as described by Kauser *et al*. (2003) (70) to measure melanin absorbance at 475 nm with a standard curve of synthetic melanin (Sigma-Aldrich, Milwaukee, USA) from 1-100 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. Two sample t-tests assuming unequal variances were performed to determine differences between the mean melanin contents of wild type strains and each of the three mutants for *C. beticola* and *E. fawcettii*, using a P-value of 0.05 as the significance threshold.

Acknowledgments

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Supplementary data

Suppl. Table 1. List of the polyketide synthase (PKS) accession codes used in this study.

protein_id	start KS domain	stop KS domain	Protein ID (Fig. 3)	NR-PKS	Collemare_ etal_2014	Species
AAB08104.3	12	386	AAB08104.3/12-386		1	*Bipolaris maydis*
AAC39471.1	376	751	AAC39471.1/376-751	1	1	*Aspergillus fumigatus*
AAC49191.1	381	757	AAC49191.1/381-757	1	1	*Aspergillus nidulans*
AAD31436.3	367	743	AAD31436.3/367-743	1	1	*Exophiala dermatitidis*
AAD34559.1	11	380	AAD34559.1/11-380		1	*Aspergillus terreus*
AAD38786.1	383	759	AAD38786.1/383-759	1	1	*Nodulisporium sp. ATCC74245*
AAK48943.1	33	406	AAK48943.1/33-406		1	*Byssochlamys nivea*
AAN59953.1	379	754	AAN59953.1/379-754	1	1	*Glarea lozoyensis*
AAR92208.1	5	382	AAR92208.1/5-382		1	*Fusarium verticillioides*
AAS48892.1	363	738	AAS48892.1/363-738	1	1	*Nectria haematococca*
AAS90093.1	372	748	AAS90093.1/372-748	1	1	*Aspergillus flavus*
AAS98200.1	29	402	AAS98200.1/29-402		1	*Aspergillus ochraceus*
AAT28740.1	10	387	AAT28740.1/10-387		1	*Fusarium verticillioides*
AAT69682.1	382	760	AAT69682.1/382-760	1	1	*Cercospora nicotianae*
AAU10633.1	380	758	AAU10633.1/380-758	1	1	*Fusarium graminearum*
AAX09990.1	135	712	AAX09990.1/135-712		1	*Bipolaris maydis*
ABA02239.1	50	435	ABA02239.1/50-435		1	*Monascus pilosus*
ABA02240.1	11	380	ABA02240.1/11-380		1	*Monascus pilosus*
ABB76806.1	11	387	ABB76806.1/11-387		1	*Bipolaris maydis*
ABB90282.1	365	738	ABB90282.1/365-738	1	1	*Fusarium graminearum*
ABB90283.1	10	376	ABB90283.1/10-376		1	*Fusarium graminearum*
ABS87601.1	9	391	ABS87601.1/9-391		1	*Aspergillus fumigatus Af293*
ABU63483.1	375	751	ABU63483.1/375-751	1	1	*Elsinoe fawcettii*
ACD39762.1	366	739	ACD39762.1/366-739	1	1	*Hypomyces subiculosus*
ACD39767.1	10	384	ACD39767.1/10-384		1	*Hypomyces subiculosus*
ACS68554.1	9	389	ACS68554.1/9-389		1	*Metarhizium anisopliae*
ACZ57548.1	48	426	ACZ57548.1/48-426		1	*Alternaria brassicicola*
ADY00130.1	360	725	ADY00130.1/360-725		1	*Penicillium brevicompactum*
AFP89389.1	370	746	AFP89389.1/370-746	1		*Cladosporium phlei*
AGO86662.1	5	385	AGO86662.1/5-385		1	*Fusarium heterosporum*
BAA18956.1	382	758	BAA18956.1/382-758	1	1	*Colletotrichum lagenaria*
BAC20564.1	9	394	BAC20564.1/9-394		1	*Penicillium citrinum*
BAC20566.1	30	399	BAC20566.1/30-399		1	*Penicillium citrinum*
BAD44749.1	383	747	BAD44749.1/383-747		1	*Monascus purpureus*

BAD83684.1	3	377	BAD83684.1/3-377		1	*Alternaria solani*
BAE06845.2	146	729	BAE06845.2/146-729		1	*Epichloe festucae*
BAI43678.1	3	383	BAI43678.1/3-383		1	*Aspergillus flavus*
BAK26562.1	3	383	BAK26562.1/3-383		1	*Aspergillus oryzae*
Bcin01p00060.1	6	387	Bcin01p00060.1/6-387			*Botrytis cinerea*
Bcin01p00090.1	5	377	Bcin01p00090.1/5-377			*Botrytis cinerea*
Bcin01p00440.1	116	430	Bcin01p00440.1/116-430			*Botrytis cinerea*
Bcin01p11550.1	7	386	Bcin01p11550.1/7-386			*Botrytis cinerea*
Bcin02p01680.1	7	379	Bcin02p01680.1/7-379			*Botrytis cinerea*
Bcin02p08770.1	382	758	Bcin02p08770.1/382-758	1		*Botrytis cinerea*
Bcin02p08830.1	394	765	Bcin02p08830.1/394-765			*Botrytis cinerea*
Bcin03p02010.1	396	761	Bcin03p02010.1/396-761			*Botrytis cinerea*
Bcin03p04360.1	4	386	Bcin03p04360.1/4-386			*Botrytis cinerea*
Bcin03p08050.1	373	750	Bcin03p08050.1/373-750	1		*Botrytis cinerea*
Bcin04p00640.1	487	857	Bcin04p00640.1/487-857			*Botrytis cinerea*
Bcin04p06330.1	8	185	Bcin04p06330.1/8-185			*Botrytis cinerea*
Bcin05p06220.1	360	733	Bcin05p06220.1/360-733	1		*Botrytis cinerea*
Bcin05p08400.1	9	383	Bcin05p08400.1/9-383			*Botrytis cinerea*
Bcin07p02920.1	69	443	Bcin07p02920.1/69-443			*Botrytis cinerea*
Bcin08p00290.1	34	399	Bcin08p00290.1/34-399			*Botrytis cinerea*
Bcin10p00040.1	10	401	Bcin10p00040.1/10-401			*Botrytis cinerea*
Bcin11p02700.1	3	373	Bcin11p02700.1/3-373			*Botrytis cinerea*
Bcin13p01510.1	7	383	Bcin13p01510.1/7-383			*Botrytis cinerea*
Bcin14p00600.1	7	381	Bcin14p00600.1/7-381			*Botrytis cinerea*
Bcin14p01290.1	36	413	Bcin14p01290.1/36-413			*Botrytis cinerea*
Bcin16p01830.1	391	763	Bcin16p01830.1/391-763	1		*Botrytis cinerea*
Bcin16p02410.1	2	385	Bcin16p02410.1/2-385			*Botrytis cinerea*
Bcin16p05040.1	385	757	Bcin16p05040.1/385-757			*Botrytis cinerea*
CAA46695.2	377	752	CAA46695.2/377-752	1	1	*Aspergillus nidulans*
CAB92399.1	344	718	CAB92399.1/344-718	1	1	*Fusarium fujikuroi*
CADAFUAP 00000040	17	385	CADAFUAP00000040/17-385			*Aspergillus fumigatus*
CADAFUAP 00000167	387	759	CADAFUAP00000167/387-759			*Aspergillus fumigatus*
CADAFUAP 00000313	36	412	CADAFUAP00000313/36-412			*Aspergillus fumigatus*
CADAFUAP 00000361	112	430	CADAFUAP00000361/112-430			*Aspergillus fumigatus*
CADAFUAP 00000765	15	415	CADAFUAP00000765/15-415			*Aspergillus fumigatus*

CADAFUAP 00000790	386	751	CADAFUAP00000790/386-751			*Aspergillus fumigatus*
CADAFUAP 00000848	9	391	CADAFUAP00000848/9-391			*Aspergillus fumigatus*
CADAFUAP 00001085	390	767	CADAFUAP00001085/390-767	1		*Aspergillus fumigatus*
CADAFUAP 00001793	5	387	CADAFUAP00001793/5-387			*Aspergillus fumigatus*
CADAFUAP 00002448	392	767	CADAFUAP00002448/392-767	1		*Aspergillus fumigatus*
CADAFUAP 00003579	2	373	CADAFUAP00003579/2-373			*Aspergillus fumigatus*
CADAFUAP 00003736	11	386	CADAFUAP00003736/11-386			*Aspergillus fumigatus*
CADAFUAP 00004097	376	751	CADAFUAP00004097/376-751	1		*Aspergillus fumigatus*
CADAFUAP 00005031	67	436	CADAFUAP00005031/67-436			*Aspergillus fumigatus*
CADAFUAP 00007262	10	367	CADAFUAP00007262/10-367			*Aspergillus fumigatus*
CADAFUAP 00007993	408	786	CADAFUAP00007993/408-786	1		*Aspergillus fumigatus*
CADAFUAP 00009219	50	426	CADAFUAP00009219/50-426			*Aspergillus fumigatus*
CAG28797.1	11	395	CAG28797.1/11-395		1	*Magnaporthe grisea*
CAG28798.1	6	390	CAG28798.1/6-390		1	*Magnaporthe grisea*
CAG29113.1	12	392	CAG29113.1/12-392		1	*Magnaporthe oryzae 70-15*
CAL69597.1	16	401	CAL69597.1/16-401		1	*Beauveria bassiana*
CAM35471.1	390	766	CAM35471.1/390-766	1	1	*Sordaria macrospora*
CAO91861.1	7	386	CAO91861.1/7-386		1	*Penicillium expansum*
CAP58786.1	6	387	CAP58786.1/6-387		1	*Botrytis cinerea*
CBET3_00098-RA	382	747	CBET3_00098-RA/382-747			*Cercospora beticola*
CBET3_00833-RA	383	761	CBET3_00833-RA/383-761	1		*Cercospora beticola*
CBET3_01483-RA	6	182	CBET3_01483-RA/6-182			*Cercospora beticola*
CBET3_02338-RA	401	779	CBET3_02338-RA/401-779	1		*Cercospora beticola*
CBET3_02473-RA	385	765	CBET3_02473-RA/385-765	1		*Cercospora beticola*
CBET3_02934-RA	4	378	CBET3_02934-RA/4-378			*Cercospora beticola*
CBET3_02994-RA	371	745	CBET3_02994-RA/371-745	1		*Cercospora beticola*
CBET3_03238-RA	31	407	CBET3_03238-RA/31-407			*Cercospora beticola*
CBET3_03942-RA	1	287	CBET3_03942-RA/1-287			*Cercospora beticola*

CBET3_04450-RA	8	182	CBET3_04450-RA/8-182			*Cercospora beticola*
CBET3_04827-RA	371	747	CBET3_04827-RA/371-747	1		*Cercospora beticola*
CBET3_04929-RA	10	188	CBET3_04929-RA/10-188			*Cercospora beticola*
CBET3_05289-RA	10	383	CBET3_05289-RA/10-383			*Cercospora beticola*
CBET3_06561-RA	118	436	CBET3_06561-RA/118-436			*Cercospora beticola*
CBET3_06649-RA	6	383	CBET3_06649-RA/6-383			*Cercospora beticola*
CBET3_08472-RA	2	370	CBET3_08472-RA/2-370			*Cercospora beticola*
CBET3_09010-RA	9	381	CBET3_09010-RA/9-381			*Cercospora beticola*
CBET3_09638-RA	370	746	CBET3_09638-RA/370-746	1		*Cercospora beticola*
CBET3_10095-RA	57	424	CBET3_10095-RA/57-424			*Cercospora beticola*
CBET3_10866-RA	15	381	CBET3_10866-RA/15-381			*Cercospora beticola*
CBET3_10910-RA	389	765	CBET3_10910-RA/389-765	1		*Cercospora beticola*
CBET3_11350-RA	369	743	CBET3_11350-RA/369-743	1		*Cercospora beticola*
CBET3_11784-RA	3	389	CBET3_11784-RA/3-389			*Cercospora beticola*
CBX87032.1	5	358	CBX87032.1/5-358		1	*Botrytis cinerea B05.10*
CLAPHL03274-RA	7	183	CLAPHL03274-RA/7-183			*Cladosporium phlei*
CLAPHL04403-RA	115	432	CLAPHL04403-RA/115-432			*Cladosporium phlei*
CLAPHL04480-RA	404	782	CLAPHL04480-RA/404-782	1		*Cladosporium phlei*
CLAPHL05093-RA	8	389	CLAPHL05093-RA/8-389			*Cladosporium phlei*
CLAPHL05460-RA	396	771	CLAPHL05460-RA/396-771	1		*Cladosporium phlei*
CLAPHL05563-RA	33	409	CLAPHL05563-RA/33-409			*Cladosporium phlei*
CLAPHL05567-RA	10	386	CLAPHL05567-RA/10-386			*Cladosporium phlei*
CLAPHL08021-RA	6	211	CLAPHL08021-RA/6-211			*Cladosporium phlei*
CLAPHL08237-RA	813	1194	CLAPHL08237-RA/813-1194			*Cladosporium phlei*
CLAPHL08786-RA	370	746	CLAPHL08786-RA/370-746	1		*Cladosporium phlei*
CLAPHL09453-RA	6	400	CLAPHL09453-RA/6-400			*Cladosporium phlei*
CLAPHL10557-RA	1	314	CLAPHL10557-RA/1-314			*Cladosporium phlei*
CLAPHL11044-RA	39	412	CLAPHL11044-RA/39-412			*Cladosporium phlei*
CLAPHL11108-RA	12	358	CLAPHL11108-RA/12-358			*Cladosporium phlei*
EAA65602.1	436	812	EAA65602.1/436-812		1	*Aspergillus nidulans FGSC A4*
EAA65604.1	13	359	EAA65604.1/13-359		1	*Aspergillus nidulans FGSC A4*
EAT76544.2	1	257	EAT76544.2/1-257			*ParaStagonospora nodorum*
EAT76667.2	166	542	EAT76667.2/166-542	1		*ParaStagonospora nodorum*
EAT77779.2	6	374	EAT77779.2/6-374			*ParaStagonospora nodorum*
EAT79399.1	6	184	EAT79399.1/6-184			*ParaStagonospora nodorum*
EAT79697.2	1	163	EAT79697.2/1-163			*ParaStagonospora nodorum*
EAT80393.2	368	725	EAT80393.2/368-725	1		*ParaStagonospora nodorum*
EAT80980.2	19	320	EAT80980.2/19-320			*ParaStagonospora nodorum*
EAT81575.2	80	452	EAT81575.2/80-452			*ParaStagonospora nodorum*

EAT82267.2	110	486	EAT82267.2/110-486	1		*ParaStagonospora nodorum*
EAT82755.2	1	262	EAT82755.2/1-262			*ParaStagonospora nodorum*
EAT82888.2	51	420	EAT82888.2/51-420			*ParaStagonospora nodorum*
EAT83281.2	59	371	EAT83281.2/59-371			*ParaStagonospora nodorum*
EAT83782.2	166	548	EAT83782.2/166-548	1		*ParaStagonospora nodorum*
EAT84550.2	20	397	EAT84550.2/20-397	1		*ParaStagonospora nodorum*
EAT85332.2	9	362	EAT85332.2/9-362			*ParaStagonospora nodorum*
EAT85671.2	333	703	EAT85671.2/333-703			*ParaStagonospora nodorum*
EAT86513.1	345	666	EAT86513.1/345-666			*ParaStagonospora nodorum*
EAT87259.2	5	369	EAT87259.2/5-369			*ParaStagonospora nodorum*
EAT87501.1	6	184	EAT87501.1/6-184			*ParaStagonospora nodorum*
EAT89292.2	1	171	EAT89292.2/1-171			*ParaStagonospora nodorum*
EAT90378.1	116	432	EAT90378.1/116-432			*ParaStagonospora nodorum*
EAT91803.2	11	358	EAT91803.2/11-358			*ParaStagonospora nodorum*
EAT91972.1	1	348	EAT91972.1/1-348			*ParaStagonospora nodorum*
EAT92602.2	75	388	EAT92602.2/75-388			*ParaStagonospora nodorum*
EAU38971.1	5	385	EAU38971.1/5-385		1	*Aspergillus terreus NIH2624*
EEP78969.1	12	394	EEP78969.1/12-394		1	*Uncinocarpus reesii 1704*
EGU88865.1	5	382	EGU88865.1/5-382		1	*Fusarium oxysporum Fo5176*
EHA28527.1	376	751	EHA28527.1/376-751	1	1	*Aspergillus niger ATCC 1015*
ELSFAW00772-RA	118	435	ELSFAW00772-RA/118-435			*Elsinoë fawcettii*
ELSFAW04358-RA	3	388	ELSFAW04358-RA/3-388			*Elsinoë fawcettii*
ELSFAW04473-RA	6	211	ELSFAW04473-RA/6-211			*Elsinoë fawcettii*
ELSFAW04998-RA	6	380	ELSFAW04998-RA/6-380			*Elsinoë fawcettii*
ELSFAW05017-RA	85	451	ELSFAW05017-RA/85-451			*Elsinoë fawcettii*
ELSFAW06585-RA	1	294	ELSFAW06585-RA/1-294			*Elsinoë fawcettii*
ELSFAW07673-RA	380	758	ELSFAW07673-RA/380-758	1		*Elsinoë fawcettii*
ELSFAW08003-RA	382	761	ELSFAW08003-RA/382-761	1		*Elsinoë fawcettii*
ELSFAW09157-RA	375	751	ELSFAW09157-RA/375-751	1		*Elsinoë fawcettii*
EMD84807	3	377	EMD84807/3-377			*Cochliobolus heterostrophus*
EMD84907	3	377	EMD84907/3-377			*Cochliobolus heterostrophus*
EMD85328	213	582	EMD85328/213-582			*Cochliobolus heterostrophus*
EMD85570	25	402	EMD85570/25-402			*Cochliobolus heterostrophus*
EMD85852	2	379	EMD85852/2-379			*Cochliobolus heterostrophus*
EMD87534	33	404	EMD87534/33-404			*Cochliobolus heterostrophus*
EMD88374	6	377	EMD88374/6-377			*Cochliobolus heterostrophus*
EMD88531	7	185	EMD88531/7-185			*Cochliobolus heterostrophus*
EMD88582	70	441	EMD88582/70-441			*Cochliobolus heterostrophus*
EMD89014	112	426	EMD89014/112-426			*Cochliobolus heterostrophus*
EMD89247	7	351	EMD89247/7-351			*Cochliobolus heterostrophus*

EMD89515	15	358	EMD89515/15-358	1		*Cochliobolus heterostrophus*
EMD90232	21	395	EMD90232/21-395			*Cochliobolus heterostrophus*
EMD90816	6	383	EMD90816/6-383			*Cochliobolus heterostrophus*
EMD91115	13	385	EMD91115/13-385			*Cochliobolus heterostrophus*
EMD92722	2	379	EMD92722/2-379			*Cochliobolus heterostrophus*
EMD93030	19	389	EMD93030/19-389			*Cochliobolus heterostrophus*
EMD93080	7	377	EMD93080/7-377			*Cochliobolus heterostrophus*
EMD93081	375	755	EMD93081/375-755			*Cochliobolus heterostrophus*
EMD93171	9	388	EMD93171/9-388			*Cochliobolus heterostrophus*
EMD93898	388	767	EMD93898/388-767	1		*Cochliobolus heterostrophus*
EMD94543	459	827	EMD94543/459-827			*Cochliobolus heterostrophus*
EMD95112	3	377	EMD95112/3-377			*Cochliobolus heterostrophus*
EMD96875	371	747	EMD96875/371-747	1		*Cochliobolus heterostrophus*
EMD97689	6	184	EMD97689/6-184			*Cochliobolus heterostrophus*
EMD97890	18	388	EMD97890/18-388			*Cochliobolus heterostrophus*
EMD97899	649	1023	EMD97899/649-1023			*Cochliobolus heterostrophus*
EME39092.1	373	749	EME39092.1/373-749	1	1	*Dothistroma septosporum NZE10*
EXF73224	7	387	EXF73224/7-387			*Colletotrichum fioriniae*
EXF74064	5	385	EXF74064/5-385			*Colletotrichum fioriniae*
EXF75718	7	386	EXF75718/7-386			*Colletotrichum fioriniae*
EXF75878	50	428	EXF75878/50-428			*Colletotrichum fioriniae*
EXF76094	2	376	EXF76094/2-376			*Colletotrichum fioriniae*
EXF76712	391	769	EXF76712/391-769	1		*Colletotrichum fioriniae*
EXF77318	7	401	EXF77318/7-401			*Colletotrichum fioriniae*
EXF77645	5	376	EXF77645/5-376			*Colletotrichum fioriniae*
EXF77657	18	388	EXF77657/18-388			*Colletotrichum fioriniae*
EXF77788	16	383	EXF77788/16-383			*Colletotrichum fioriniae*
EXF77798	116	434	EXF77798/116-434			*Colletotrichum fioriniae*
EXF77954	7	309	EXF77954/7-309			*Colletotrichum fioriniae*
EXF78137	202	572	EXF78137/202-572	1		*Colletotrichum fioriniae*
EXF79058	3	377	EXF79058/3-377			*Colletotrichum fioriniae*
EXF79508	8	290	EXF79508/8-290			*Colletotrichum fioriniae*
EXF79511	30	404	EXF79511/30-404			*Colletotrichum fioriniae*
EXF79648	17	381	EXF79648/17-381			*Colletotrichum fioriniae*
EXF80059	382	758	EXF80059/382-758	1		*Colletotrichum fioriniae*
EXF80071	11	383	EXF80071/11-383			*Colletotrichum fioriniae*
EXF80297	9	382	EXF80297/9-382			*Colletotrichum fioriniae*
EXF80380	2	384	EXF80380/2-384			*Colletotrichum fioriniae*
EXF80608	15	389	EXF80608/15-389			*Colletotrichum fioriniae*
EXF84093	400	780	EXF84093/400-780	1		*Colletotrichum fioriniae*

EXF85208	17	403	EXF85208/17-403			*Colletotrichum fioriniae*
EXF85213	58	410	EXF85213/58-410			*Colletotrichum fioriniae*
EXF85322	10	394	EXF85322/10-394			*Colletotrichum fioriniae*
EXF85385	90	464	EXF85385/90-464			*Colletotrichum fioriniae*
EXF85533	10	188	EXF85533/10-188			*Colletotrichum fioriniae*
EXF86307	367	742	EXF86307/367-742	1		*Colletotrichum fioriniae*
EXF86315	10	384	EXF86315/10-384			*Colletotrichum fioriniae*
ACB12550.1	30	401	ACB12550.1/30-401		1	*Fusarium oxysporum*
MGG_00233T0	11	384	MGG_00233T0/11-384			*Magnaporthe oryzae*
MGG_00241T0	380	751	MGG_00241T0/380-751	1		*Magnaporthe oryzae*
MGG_00428T0	412	789	MGG_00428T0/412-789	1		*Magnaporthe oryzae*
MGG_00806T0	156	443	MGG_00806T0/156-443			*Magnaporthe oryzae*
MGG_03810T0	12	395	MGG_03810T0/12-395			*Magnaporthe oryzae*
MGG_04775T0	62	333	MGG_04775T0/62-333			*Magnaporthe oryzae*
MGG_05589T0	66	441	MGG_05589T0/66-441			*Magnaporthe oryzae*
MGG_07219T0	388	764	MGG_07219T0/388-764	1		*Magnaporthe oryzae*
MGG_07803T0	1263	1660	MGG_07803T0/1263-1660			*Magnaporthe oryzae*
MGG_08236T0	7	380	MGG_08236T0/7-380			*Magnaporthe oryzae*
MGG_08281T0	46	470	MGG_08281T0/46-470	1		*Magnaporthe oryzae*
MGG_08285T0	12	364	MGG_08285T0/12-364			*Magnaporthe oryzae*
MGG_09589T0	3	412	MGG_09589T0/3-412			*Magnaporthe oryzae*
MGG_09645T0	51	415	MGG_09645T0/51-415			*Magnaporthe oryzae*
MGG_10011T0	404	776	MGG_10011T0/404-776			*Magnaporthe oryzae*
MGG_10202T0	4	407	MGG_10202T0/4-407			*Magnaporthe oryzae*
MGG_10912T0	52	425	MGG_10912T0/52-425			*Magnaporthe oryzae*
MGG_11638T0	9	395	MGG_11638T0/9-395			*Magnaporthe oryzae*
MGG_12154T0	116	433	MGG_12154T0/116-433			*Magnaporthe oryzae*
MGG_12214T0	7	378	MGG_12214T0/7-378			*Magnaporthe oryzae*
MGG_12447T0	11	395	MGG_12447T0/11-395			*Magnaporthe oryzae*
MGG_12613T0	19	385	MGG_12613T0/19-385			*Magnaporthe oryzae*
MGG_13591T0	1	311	MGG_13591T0/1-311			*Magnaporthe oryzae*
MGG_13767T0	9	388	MGG_13767T0/9-388			*Magnaporthe oryzae*
MGG_14831T0	36	410	MGG_14831T0/36-410			*Magnaporthe oryzae*
MGG_14897T0	2	391	MGG_14897T0/2-391			*Magnaporthe oryzae*
MGG_14943T0	9	366	MGG_14943T0/9-366			*Magnaporthe oryzae*
MGG_14945T0	107	481	MGG_14945T0/107-481			*Magnaporthe oryzae*
MGG_15097T0	6	390	MGG_15097T0/6-390			*Magnaporthe oryzae*
MGG_15100T0	12	393	MGG_15100T0/12-393			*Magnaporthe oryzae*
MGG_15272T0	4	381	MGG_15272T0/4-381			*Magnaporthe oryzae*
MGG_18133T0	1	314	MGG_18133T0/1-314	1		*Magnaporthe oryzae*
OAG13303	19	397	OAG13303/19-397			*Alternaria alternata*

OAG13655	47	418	OAG13655/47-418			*Alternaria alternata*
OAG13710	7	185	OAG13710/7-185			*Alternaria alternata*
OAG14483	7	382	OAG14483/7-382			*Alternaria alternata*
OAG15814	1	326	OAG15814/1-326			*Alternaria alternata*
OAG16734	9	374	OAG16734/9-374			*Alternaria alternata*
OAG17698	1129	1501	OAG17698/1129-1501			*Alternaria alternata*
OAG18296	6	184	OAG18296/6-184			*Alternaria alternata*
OAG18885	2	376	OAG18885/2-376			*Alternaria alternata*
OAG18929	5	383	OAG18929/5-383			*Alternaria alternata*
OAG22978	21	395	OAG22978/21-395			*Alternaria alternata*
OAG24024	116	430	OAG24024/116-430			*Alternaria alternata*
OAG24502	371	747	OAG24502/371-747	1		*Alternaria alternata*
OAG24819	139	515	OAG24819/139-515	1		*Alternaria alternata*
OAG25652	259	629	OAG25652/259-629			*Alternaria alternata*
OAG26059	2	382	OAG26059/2-382			*Alternaria alternata*
P22367.1	33	406	P22367.1/33-406		1	*Penicillium griseofulvum*
Q9Y8A5.1	9	394	Q9Y8A5.1/9-394		1	*Aspergillus terreus*
SHIR00028-RA	14	394	SHIR00028-RA/14-394			*Shiraia sp.*
SHIR00389-RA	390	757	SHIR00389-RA/390-757			*Shiraia sp.*
SHIR00708-RA	53	427	SHIR00708-RA/53-427			*Shiraia sp.*
SHIR01733-RA	14	394	SHIR01733-RA/14-394			*Shiraia sp.*
SHIR02320-RA	372	748	SHIR02320-RA/372-748	1		*Shiraia sp.*
SHIR02347-RA	36	409	SHIR02347-RA/36-409			*Shiraia sp.*
SHIR02657-RA	14	337	SHIR02657-RA/14-337			*Shiraia sp.*
SHIR02743-RA	55	432	SHIR02743-RA/55-432			*Shiraia sp.*
SHIR03412-RA	1	158	SHIR03412-RA/1-158			*Shiraia sp.*
SHIR04032-RA	116	434	SHIR04032-RA/116-434			*Shiraia sp.*
SHIR05834-RA	9	383	SHIR05834-RA/9-383			*Shiraia sp.*
SHIR05992-RA	259	636	SHIR05992-RA/259-636	1		*Shiraia sp.*
SHIR06023-RA	11	393	SHIR06023-RA/11-393			*Shiraia sp.*
SHIR06623-RA	367	745	SHIR06623-RA/367-745	1		*Shiraia sp.*
SHIR06625-RA	1	286	SHIR06625-RA/1-286			*Shiraia sp.*
SHIR06679-RA	372	747	SHIR06679-RA/372-747	1		*Shiraia sp.*
SHIR08141-RA	14	392	SHIR08141-RA/14-392			*Shiraia sp.*
SHIR08147-RA	394	769	SHIR08147-RA/394-769	1		*Shiraia sp.*
SHIR08477-RA	373	755	SHIR08477-RA/373-755	1		*Shiraia sp.*
SHIR08754-RA	396	774	SHIR08754-RA/396-774	1		*Shiraia sp.*
SHIR08896-RA	112	486	SHIR08896-RA/112-486			*Shiraia sp.*
SHIR09115-RA	7	185	SHIR09115-RA/7-185			*Shiraia sp.*
SHIR10307-RA	12	387	SHIR10307-RA/12-387			*Shiraia sp.*
SHIR10308-RA	383	756	SHIR10308-RA/383-756	1		*Shiraia sp.*

XP_001217072.1	403	781	XP_001217072.1/403-781	1	1	*Aspergillus terreus NIH2624*
XP_001221381.1	14	396	XP_001221381.1/14-396		1	*Chaetomium globosum CBS 148.51*
XP_001242733.1	11	387	XP_001242733.1/11-387		1	*Coccidioides immitis RS*
XP_001269050.1	5	383	XP_001269050.1/5-383		1	*Aspergillus clavatus NRRL 1*
XP_001270543.1	9	393	XP_001270543.1/9-393		1	*Aspergillus clavatus NRRL 1*
XP_001790998.1	11	358	XP_001790998.1/11-358		1	*Phaeosphaeria nodorum SN15*
XP_001910795.1	382	758	XP_001910795.1/382-758	1	1	*Podospora anserina S mat+*
XP_003715434.1	388	764	XP_003715434.1/388-764	1	1	*Magnaporthe oryzae 70-15*
XP_390640.1	7	366	XP_390640.1/7-366		1	*Fusarium graminearum PH-1*
XP_657754.1	417	795	XP_657754.1/417-795	1	1	*Aspergillus nidulans FGSC A4*
XP_681681.1	3	388	XP_681681.1/3-388		1	*Aspergillus nidulans FGSC A4*
XP_746435.1	392	767	XP_746435.1/392-767	1	1	*Aspergillus fumigatus Af293*
XP_748662.1	138	723	XP_748662.1/138-723		1	*Aspergillus fumigatus Af293*

Supl. Table 2. Primers used in this study

Primer	Specificity/role	Sequence (5' to 3')
MDB-277	Split-marker M13F (HYG-F)	GACGTTGTAAAACGACGGCCAGTG
MDB-258	Split-marker: HY (NLC37) (HY-R)	GGATGCCTCCGCTCGAAGTA
MDB-259	Split-marker: YG (NLC38) (YG-F)	CGTTGCAAGACCTGCCTGAA
MDB-278	Split-marker M13R (HYG-R)	CACAGGAAACAGCTATGACCATGA
MDB-1145	HY-R2 (split marker)	GGCAGGTAGATGACGACCAT
MDB-1598	Ef ELS PKS 1F	CGAACCGAGCAACAGTGATA
MDB-1599	Ef ELS PKS 2R	CACTGGCCGTCGTTTTACAACGTCACGGAGATTCTGGCTGCTTA
MDB-1600	Ef ELS PKS 3F	TCATGGTCATAGCTGTTTCCTGTGCACCTGGTAGAAGGCGCTAC
MDB-1601	Ef ELS PKS 4R	TCAACATGCTGACAGATTGC
MDB-1602	Ef ELS PKS 5'1F	GGAGTCCAGAGATCCGACTG
MDB-1603	Ef MEL PKS 1F	ACGCTGCACATGTTATCGAG
MDB-1604	Ef MEL PKS 2R	CACTGGCCGTCGTTTTACAACGTCCTTCTTGACGGGGTATCGAA
MDB-1605	Ef MEL PKS 3F	TCATGGTCATAGCTGTTTCCTGTGGGAGTCGAGAGGGAAAGGTC
MDB-1606	Ef MEL PKS 4R	GCCATGTAGAGGAGGTGGAA
MDB-1607	Ef MEL PKS 5'1F	GTCACGTCGAGTCCACACAC
MKE-177	Ef Mel gene spec Fp	TGGGTACAACGTGGCTCATA
MKE-178	Ef Mel gene spec Rp	GACGATGAAGCCACCAAGAT
MKE-179	Ef Els gene spec Fp	CAAGGAACAAATGCAGAGCA
MKE-180	Ef Els gene spec Rp	GAGCCGACTCAAAATCCTTG
MDB-1444	Cb CTB1 1F	TCCTCTGGTGCTATGTCACG
MDB-1445	Cb CTB1 2R	CACTGGCCGTCGTTTTACAACGTCGAGATGGCAGAGGTACAGCT
MDB-1446	Cb CTB1 3F	TCATGGTCATAGCTGTTTCCTGTGTAACTCCGTCTCCAACCACC
MDB-1447	Cb CTB1 4R	CTGGTCGAGAAACTTGTGCA
MDB-1452	Cb CTB1 5'1F	GAGCGTGCTGTTTCCCTATG

MDB-1623	Cb MEL PKS 1F	ATAGCAGCACCGTACCAACC
MDB-1624	Cb MEL PKS 2R	CACTGGCCGTCGTTTTACAACGTCTGTCTACAGGGAAGGGCATC
MDB-1625	Cb MEL PKS 3F	TCATGGTCATAGCTGTTTCCTGTGTGTCACGGGATAACGAACAA
MDB-1626	Cb MEL PKS 4R	AGTAAGGATTCACGCCGATG
MDB-1633	Cb MEL PKS 5'1F	ACGCAGAGTTTGTCAACACG
MDB-1253	Cb CTB1 gene spec Fp	AGATCGGGATGCCAATCGAC
MDB-1254	Cb CTB1 gene spec Rp	CAATCTCCATGAACTGCGCG
MDB-1726	Cb MEL PKS gene spec Fp	GGTAGCAGCTCCAGTTCCTG
MDB-1727	Cb MEL PKS gene spec Rp	CTCAAAATGAGCGTCGTCAA

Chapter 7

General discussion

Introduction

The hemibiotrophic fungus *Cercospora beticola* causes Cercospora leaf spot of sugar beet (1). Due to its incredibly destructive nature and worldwide occurrence, this disease is of great economic importance (1, 2). Primary inoculation in the field is initiated when insects, rain or air movements transfer emerging *C. beticola* conidia onto a host plant from overwintering stromata (1, 3). Once on a sugar beet leaf, the fungus enters through the stomata and starts colonizing the mesophyll asymptomatically (1). Cercospora leaf spot symptoms will appear after approximately two weeks, depending on environmental conditions (1, 4), likely due to a switch from biotrophic to necrotrophic life style of the fungus.

For full virulence, pathogenic microbes must bypass the innate immune system of their desired host plant. This immune system depends on recognition of pathogenic intruders by IPRs (invasion pattern receptors) (5-8). Upon successful recognition, an IP-triggered response (IPTR) is initiated to unveil microbial invasion (6). Unless the invading microbe aims to induce plant defense responses such as hypersensitive responses for its own benefit, it may use effectors to suppress IPTR. Effector proteins have been traditionally described as small, cysteine-rich proteins that are deliberately secreted by pathogens to facilitate the infection process. This definition, however, is restrictive in scope and was therefore suggested to be modified to "microbially secreted molecules that contribute to niche colonization" (9, 10). The broader definition acknowledges that other microbes, such as endophytes and mutualists, also utilize effectors (9, 10). Furthermore, it implies that molecules other than proteins such as secondary metabolites (SMs) (11-13) and small RNAs (14) can fulfill effector functions that may not be limited to the ordinary plant-microbe setting but can also act in microbial competition and nutrition acquisition (9, 10, 15).

Since our knowledge on the molecular interactions that take place during *C. beticola* colonization of sugar beet by is limited, this thesis attempts to deepen our knowledge of *C. beticola* infection strategies by investigating its effector biology.

C. beticola proteinaceous effector repertoire

Effectors are known to be employed by a broad variety of different plant pathogenic fungi to evade detection by the host during colonization. Here, the apoplastic space is one of the locations where interactions between pathogens and host first takes place. Once inside the apoplast, fungal pathogens face defense responses such as chitinases that target fungal cell walls to liberate chitin fragments. These chitin fragments may act as IPs that can be perceived by IPRs, which will lead to an increased release of chitinases into the apoplast with the aim to lyse the cell wall of the invading fungal pathogen (6, 16-19). To shield the fungal cell wall from degradation by plant chitinases, organisms such as the biotrophic fungus *Cladosporium fulvum* that causes leaf mold on tomato secrete the virulence factor CfAvr4 during infection (20). Due to its peritrophin-A (PAD) domain, a common feature of the carbohydrate-binding module family 14 (CBM14) (21), the CfAvr4 effector is able to bind to chitin in the fungal cell wall and thereby protects fungal hyphae from hydrolysis by plant chitinases (20-24). When investigating

the existence of *CfAvr4* homologs in other Dothideomycetes it was found that several fungi, including *C. beticola*, harbor a CfAvr4 homolog in their genome (25, 26). In vitro carbohydrate-affinity assays with CbAvr4 and other Dothideomycete Avr4 homologs revealed that, like CfAvr4, all Avr4 homologs are able to bind chitin (26). As chitin-binding appears to be a conserved biological trait between CfAvr4 and all Avr4 homologs, it is hypothesized that all Avr4 homologs including CbAvr4 may also share the CfAvr4 function of shielding fungal hyphae from lysis by plant chitinases due to their chitin-binding ability.

Effector protein identification through comparative genomics has served as a useful tool to detect another *C. beticola* effector named CbAve1. CbAve1 is a homolog of VdAve1 (Avirulence on Ve1 tomato), which is secreted by the vascular wilt pathogen *Verticillium dahliae*. Originally, VdAve1 was identified by comparative population genomics as a linage-specific *V. dahliae* race 1 effector that is recognized by the tomato cell surface-localized immune receptor Ve1 (27). However, in the absence of Ve1, VdAve1 was demonstrated to contribute to fungal virulence (27). Interestingly, VdAve1 homologs are wide-spread and can be found in plants as well as several other plant pathogens. Based on the observation of VdAve1 involvement in virulence, it was also tested whether fungal Ave1 homologs of *Fusarium oxysporum* (FoAve1), *Colletotrichum higginsianum* (ChAve1), and CbAve1 are expressed during infection and act as virulence factors (Chapter 3). Gene expression analysis revealed that *FoAve1* and *CbAve1* are indeed expressed during infection while *ChAve1* was not. Moreover, targeted gene replacement of *FoAve1*, *ChAve1*, and *CbAve1* and subsequent virulence assays on tomato (for Δ*FoAve1*), Arabidopsis (for Δ*ChAve1*) and sugar beet (for Δ*CbAve1*) resulted in a reduced virulence of Δ*FoAve1* and Δ*CbAve1* strains compared to their respective wild type strains. Deletion of *ChAve1* however, did not have any effect on *Co. higginsianum* virulence on Arabidopsis. While this outcome indicates that CbAve1 plays a major role in *C. beticola* virulence, the exact mechanism of how CbAve1 contributes to virulence remains unknown. While current research on VdAve1 function is on-going, potential results for VdAve1 will have to be experimentally validated for their applicability to CbAve1 as *V. dahliae* and *C. beticola* differ in many aspects such as life-style, primary infection site, and infection process.

Due to its hemibiotrophic life style, it was hypothesized that *C. beticola* also secrets effectors that promote the necrotrophic phase of its life cycle. Using a phenotype-based forward genetics approach, a proteinaceous virulence factor named CbNip1 (CbNip for *Cercospora beticola* necrosis-inducing protein 1) was identified due to its necrosis-inducing activity in sugar beet and *Nicotiana benthamiana* (Chapter 4). Interestingly, NIP1's ability to induce necrosis within 48 hours was highly regulated by light. While other necrosis-inducing proteins such as ZtNIP1 and ZtNIP2 of the wheat pathogen *Zymoseptoria tritici* have been shown to need light for full functionality (28), CbNip1 was most active in complete darkness as exposure of CbNip1-infiltrated sugar beet leaf with a 12 hour light-dark cycle led only to chlorosis formation that gradually turned necrotic over time (Chapter 4). Furthermore, CbNip1 appears to contribute to necrotic symptom development, as upregulated *CbNip1* expression *in planta* correlates with necrotic lesion appearance. Unfortunately, the mode-of-action of CbNip1 and its location during infection are currently unknown. While this is the first report

of a *C. beticola* effector that depends on darkness for full activity, light-dependent secondary metabolite (SM) effectors have been known for decades to be part of the *C. beticola* armory. These SM effectors are discussed below.

C. beticola secondary metabolites

Generally fungal SMs are low molecular mass molecules that have no direct contribution to fungal development and reproduction. However, many SMs possess a bioactive nature which is of great value for the producing fungus. The intrinsic modes of action of SMs are remarkably diverse. While some SMs can help with uptake, transport and/or solubilization of metal ions, others are capable of protecting the producing fungus against abiotic and biotic stresses (13). In case of symbiotic fungi, this protection can be extended to also shield the host plant from secondary colonization by other microorganisms. Besides their useful protective function, some SMs of plant pathogenic fungi however are known to play a key role in the fungal infection process and virulence. Classification of SMs that act as toxins can be based on their toxicity range. While host-selective toxins require their host to have a specific target in order to be effective, others exhibit toxicity to a broad spectrum of different organisms and are described as non-host-selective toxins (29). For example, the hemibiotrophic pine needle pathogen *Dothistroma septosporum* is known to produce the non-host-selective secondary metabolite dothistromin (30). Dothistromin belongs to the anthraquinone family and was shown to contribute to fungal virulence (12). Similar to *D. septosporum*, *C. beticola* is also known to produce non-hosts specific toxins, namely beticolins that belong to the xanthraquinone family (31) and the perylenequinone cercosporin (32).

Beticolins

Beticolins are a group of non-host specific phytotoxins of which 20 members (B0 to B19) have been identified to date to be produced by *C. beticola* (33-35) and the hoary alyssum (*Berteroa incana*) pathogen *Cercospora berteroae* (36). Alternative names for these toxins, such as Gelbe Fraktion (GF) (37), Cercospora beticola toxin (CBT) (36), and cebetins (38, 39) arose due to simultaneous research efforts by different groups and limited data concerning their structure during early research. Later, analyses of their chemical structures revealed that beticolins are structurally closely related (Fig. 1). All have a chlorine atom attached to the central aromatic ring, while their octocyclic basic structure is composed of two subunits; a partially hydrogenated anthraquinone and a partially hydrogenated xanthone that are connected through a seven-membered ring (31, 33, 34, 40-42). Structural differences between beticolins are due to different isomeric configurations (*ortho-*, *para-*, or *epi-ortho-*) and by variable residues (31, 43, 44) (Fig. 1). Interestingly, beticolins are able to switch isomery, for example *ortho*-beticolin B2 is able to transform into the *para*-beticolin B1 or *epi-ortho*-beticolin B6 and vice versa (42) (Fig. 1). Early research on their biological function indicated that beticolins have antibacterial and phytotoxic properties (37). However, necrosis formation in plants upon beticolin application was only induced in the presence of light. Later it was found that due to their ability to form complexes with Mg^{2+}, beticolins inhibit tumoral cell growth in mice (45, 46), interfere with H^{+}-ATPase activity (40, 47-49) and are able to incorporate themselves into lipid bilayers to form

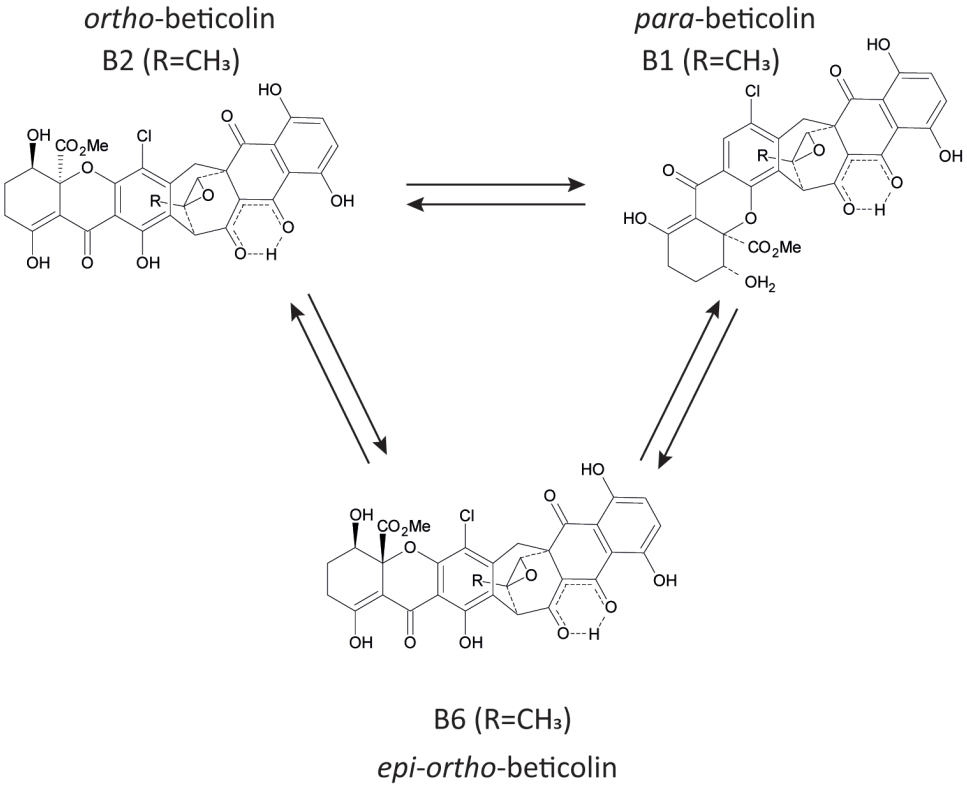

Figure 1. Beticolin structures and isoforms. Beticolins are structurally related but can differ by residues (R) and isoforms (*ortho*-, *epi-ortho*-, or *para*-beticolin). Beticolins that carry the same residues are able to transform into each other by switching isomery. For example, the *ortho*-beticolin B2 that has the the oxygen in ortho position of the clorine atom can transform into the *epi-ortho*-beticolin B6 or *para*-beticolin B1 (clorine and oxygen are in *para* position).

ion channels with poor ion selectivity (33, 34, 50). The latter property led to the classification of beticolins as ion channel-forming toxins (33). While chemical structures and biological activity have been evaluated throughout the last decades, the biosynthetic pathway of these toxins is unknown. Therefore, it is currently not possible to assess to what extent beticolin production and associated phytotoxic effects contribute to *C. beticola* virulence.

Cercosporin

The most prominent and the best-studied example of a non-host selective *C. beticola* toxin is cercosporin. Cercosporin is produced by most *Cercospora* species and belongs to the perylenequinone family. Mutant lines that are unable to produce the toxin experience a virulence penalty, indicating that cercosporin is a virulence factor for the species tested (51-53). Toxin production is light-depended as light triggers the induction of the biosynthetic genes responsible for cercosporin formation (11). These genes are organized in a cercosporin toxin biosynthesis

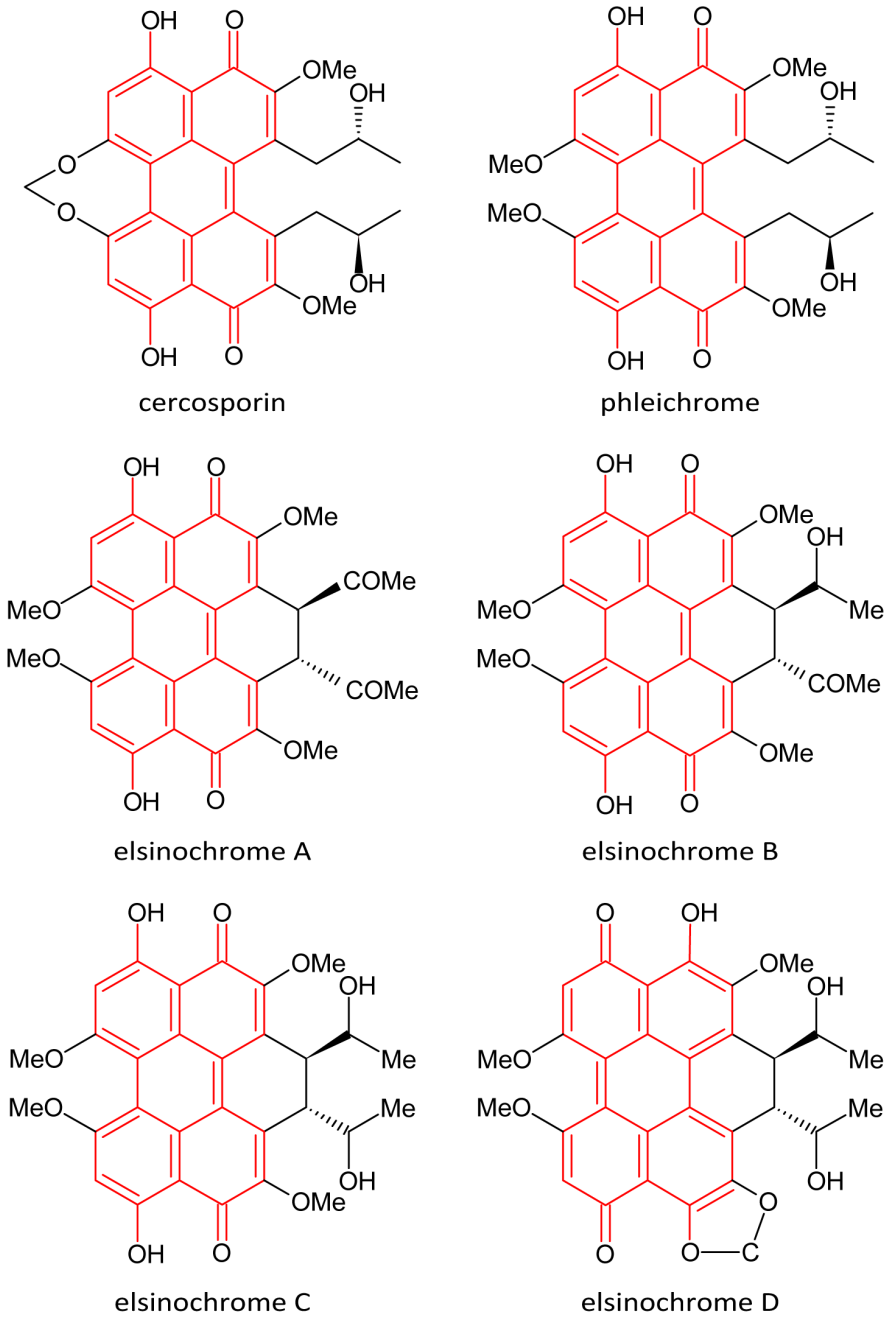

Figure 2. Structures of related perylenequinones. All perylenequinones have a common backbone structure (indicated in red) and are therefore structurally related. Structural differences between the molecules are due to differing side chains as can be observed for cercosporin secreted by *C. beticola*, phleichrome by *C. phlei* and elsinochromes A, B, C, and D produced by *E. fawcettii*.

cluster (CTB) that was shown to have experienced duplications and multiple horizontal gene transfers during evolution (Chapter 5). Although cercosporin was thought to be a unique feature of fungi that belong to the *Cercospora* genus, in Chapter 5 we show that the CTB cluster is actually remarkably widely-distributed, including many *Colletotrichum* species of which *Colletotrichum fioriniae* has been confirmed to also produce this potent toxin. Due to their indistinctive toxicity, cercosporin and other perylenequinone family members are highly harmful to nearly every living organism (11). In the following section, I will elucidate pathway genes, mode-of-action, and auto-resistance of this destructive-natured but incredibly fascinating perylenequinone toxin and other perylenequinone family members.

Perylenequinone pathway genes

All perylenequinones are structurally related. Their common feature is a 3,10-dihydroxy-4,9-perylenequinone backbone to which every perylenequinone derivate has their own combination of distinct side chains attached (54) (Fig. 2). These side chain variations arise because individual perylenequinone pathways have their own set of decorating enzymes. However, the fact that there are commonalities between perylenequinone structures suggests that also the biosynthetic pathways display significant similarity (Fig. 3). While extensive research on the CTB pathway has shed some light on some putative pathway steps (Fig. 5), it is not possible to determine a full biosynthesis scheme yet due to extreme instability of most pathway intermediates and the potential occurrence of feedback inhibition. Since every CTB gene has an alphanumerical gene name in which the numbers neither represent gene order within the cluster or which step in the pathway an enzyme performs, CTB genes will be discussed in numerical order below.

CTB1 is an iterative NR-PKS and essential for cercosporin production (52, 55, 56). As PKS genes are the key enzymes for biosynthetic pathways, a *CTB1* homolog is present in all perylenequinone clusters (Chapter 5 and 6) (52, 57-59) (Fig. 3). Like all PKS genes, *CTB1* harbors multiple functional domains including a starter unit acyltransferase (SAT), a β-ketoacyl synthase (KS), a malonyl acyltransferase (MAT), a product template domain (PT), a dual-tandem acyl-carrier (ACP_2) domain, and a thioesterase (TE) domain (55) (Fig. 4). All six catalytic domains conjointly work together to form *nor*-toralactone, the first intermediate in the cercosporin assembly line (52, 55, 60). Due to its starter unit specificity, CTB1 SAT domain selectively accepts acetyl-CoA as a starter unit (60-62). For initiation of *nor*-toralactone synthesis, the SAT domain loads the starter unit onto the ACP which in turn is responsible for substrate/product shuffling between the different functional domains (Fig. 4). In a similar fashion as the SAT domain, the MAT domain supplies the ACP with six single malonyl-CoA extender units (55, 56, 60). Subsequently, the KS domain forms a polyketide chain by attaching one ketide unit at a time through catalyzing consecutive peptide bond formations. Once condensation is complete, the PT domain mediates the characteristic cyclizations and dehydrations of the linear intermediate. Once *nor*-toralactone formation is completed, the TE domain coordinates the release of the final product from CTB1. Targeted gene replacement of *CTB1* as well as of the homolog in the elsinochrome pathway (Ef*ETB1*) resulted in complete abolishment of perylenequinone formation (Chapter 5). Loss of CTB1 function and consequently the inability to produce cercosporin was reported to lead to reduced virulence in *C. beticola*, *C. nicotianae* and *C. kikutchii* (51-53).

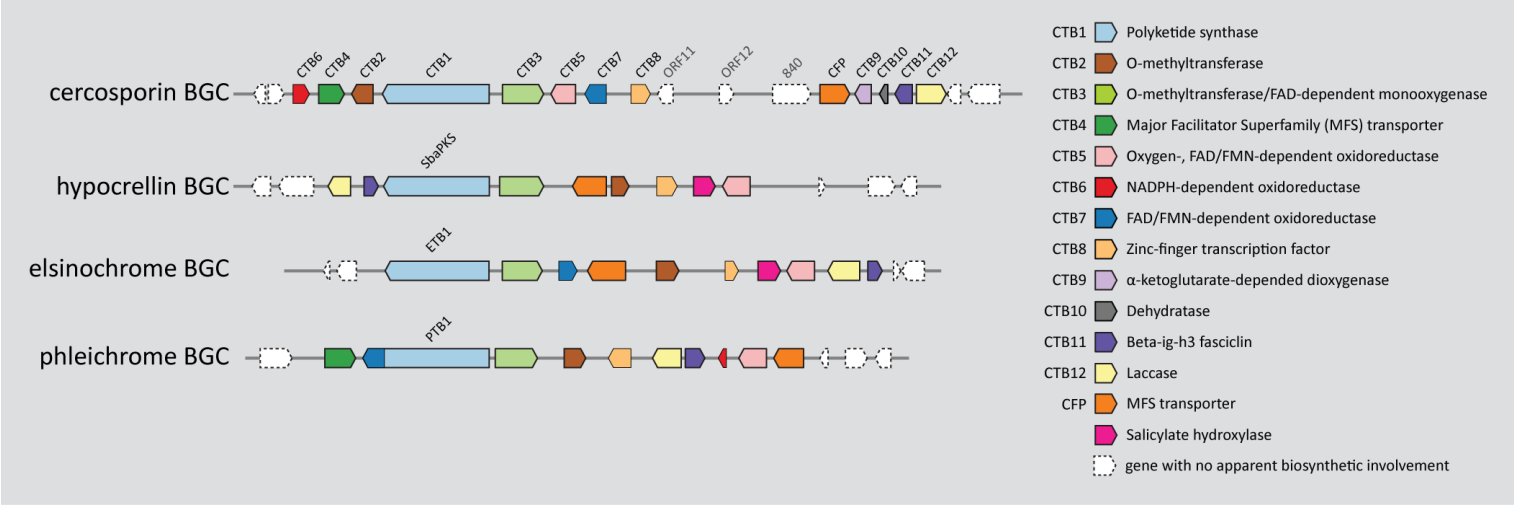

Figure 3. Gene composition scheme of known perylenequinone biosynthetic gene clusters (BGCs). On top is the cercosporin BGC used as reference and below are the hypocrellin BGC, elsinochrome BGC, and phleichrome BGC. Orthologous genes are indicated by color (color key and annotated functions can be found in the legend to the right). While the majority of orthologous genes are conserved in all clusters, each cluster has its individual combination of genes.

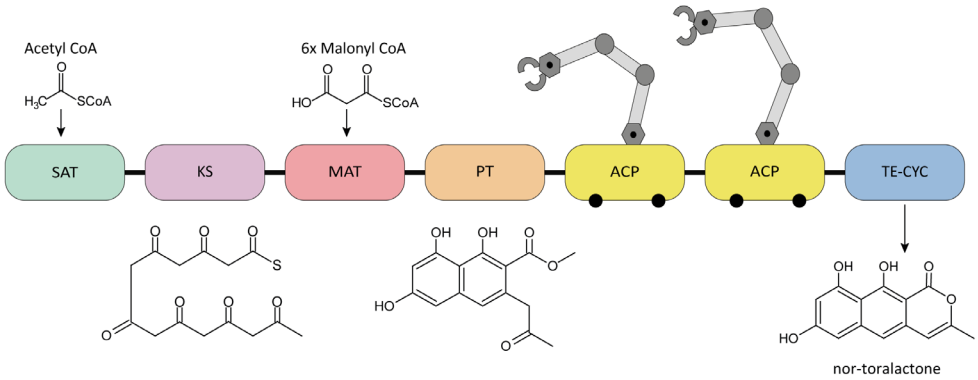

Figure 4. Multidomain structure of the PKS CTB1. Formation of *nor*-toralactone by CTB1 starts with the starter unit acyltransferase (SAT) specifically accepting acetyl-CoA. Successive condensation in the β-ketoacyl synthase (KS) domain extends this acetyl-CoA with six malonyl-CoA extender units provided by the malonyl acyltransferase (MAT). Subsequently, the product template domain (PT) will catalyze correct cyclizations and dehydrations of the linear polyketide chain. End product (*nor*-toralactone) release is mediated by the thioesterase (TE) domain. Throughout the whole process, the ACP domains are responsible for moving substrates and products from one functional domain to the other.

CTB2 is an O-methyltransferase (63) present in the elsinochrome, phleichrome, and hypocrellin clusters (Chapter 6) (Fig. 3). It is hypothesized to mediate intermediate steps of *O*-dimethylation as well as side chain ketone reduction (Fig. 5). Targeted gene deletion of *CTB2*, resulted in mutant strains that are completely lacking a metabolic profile and showed a severe virulence penalty *in planta* compared to the cercosporin-producing wild type strain (60, 63, 64).

CTB3, which is predicted as an *O*-methyltansferase FAD-depended monooxygenase, is proposed to mediate the second step of cercosporin biosynthesis using the precursor *nor*-toralactone as substrate (60) (Fig. 5). Interestingly, this enzyme has a dual function as it harbors a putative O-methyltransferase at the N-terminus a and a putative Flavin-dependent monooxygenase domain at the C-terminus (60, 65). Individual heterologous expression of each domain revealed that the O-methyltransferase domain is responsible for the conversion of *nor*-toralactone to toralactone. In turn, toralactone serves as a substrate for CTB3 Flavin-dependent monooxygenase domain which is responsible for the occurrence of an oxidative ring opening. Inoculation assays of *CTB3* disruption strains showed that lack of CTB3 impairs fungal virulence (65). Homologs of this enzyme can be found in all known biosynthetic perylenequinone pathways (Chapter 6) (Fig. 3).

CTB4 is a major facilitator superfamily (MFS) transmembrane transporter (64, 66). *C. beticola CTB4* disruption strains displayed impairment in cercosporin production by at least 35% (66). Since cercosporin produced by the *CTB4* mutants accumulated in the fungal mycelium and was not secreted into the medium, Choquer *et al.* (2007) (66) suggested that the lack of CTB4 transporter function impaired cercosporin secretion. However, when stimulated by high light conditions, *CTB4* mutants secreted a dark brown compound of unknown nature that quickly diffused into the solid medium (66). Interestingly, a *CTB4* homolog is also present in the putative phleichrome biosynthetic

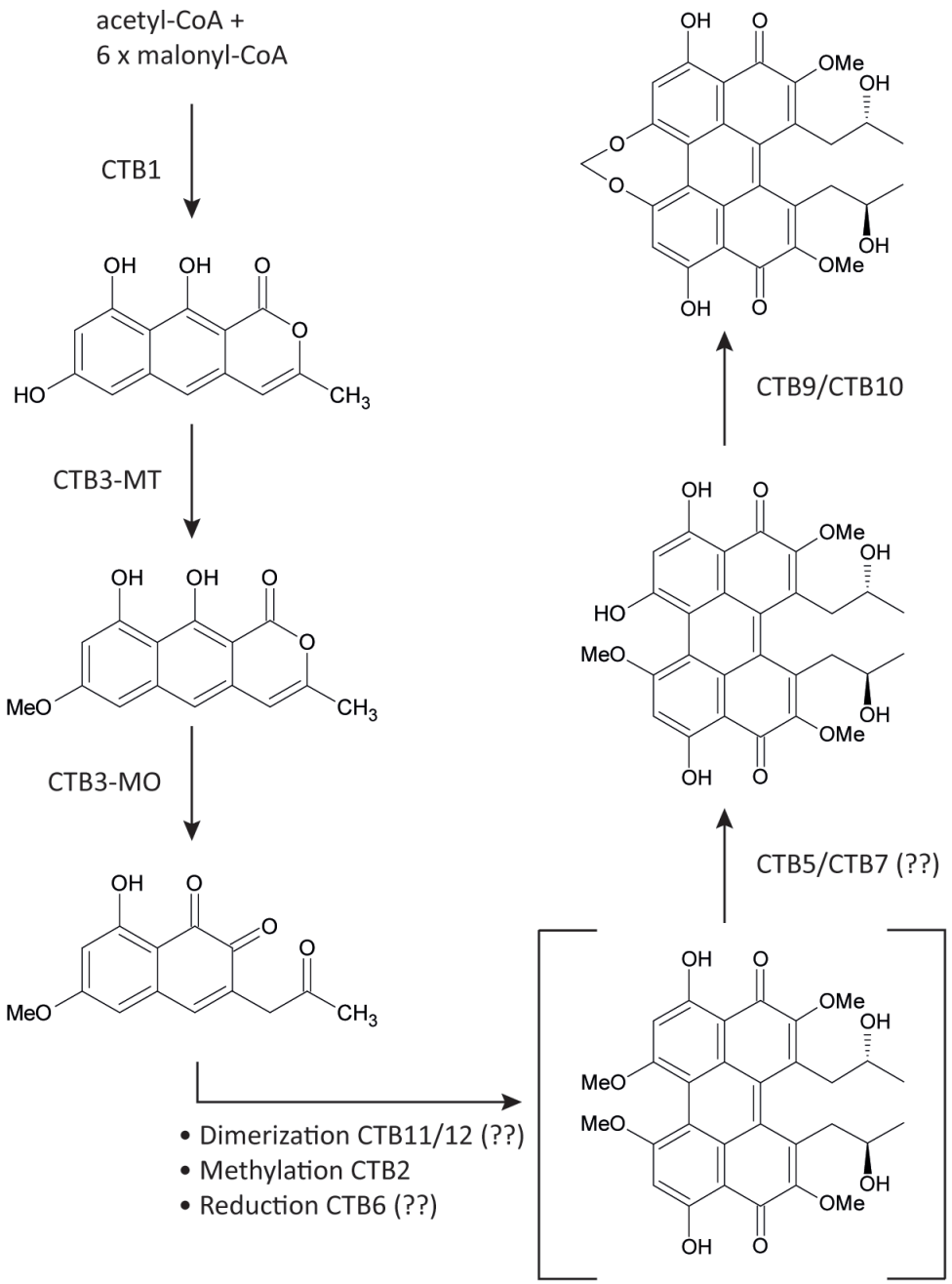

Figure 5. Preliminary scheme of the cercosporin biosynthetic pathway consisting of 12 cluster genes. CTB1 forms *nor*-toralactone which is processed to cercosquinone B by CTB3 methyltransferase (CTB3-MT) and monooxygenase CTB3-MO). Further processing of this cercosporin intermediate might be mediated by CTB2, CTB6, and CTB10 and CTB11 to yield the cercosporin intermediate displayed in a square bracket which has not been directly observed but is rather logically inferred. CTB5 or CTB7 are hypothesized to prime the cercosporin molecule for methylenedioxy bridge formation by CTB9 and CTB10. (Scheme was adopted from chapter 5)

pathway but missing in the predicted elsinochrome and hypocrellin clusters (Chapter 5) (Fig. 3). As elsinochrome and hypocrellin are secreted by *E. fawcettii* and *S. bambusicola* respectively despite the lack of a *CTB4* homolog, the question arises whether CTB4 is indeed solely responsible for toxin export in *C. nicotianae* as suggested by Choquer *et al*. (2007) (66) or whether other transporter proteins can functionally substitute toxin secretion in the absence of CTB4.

CTB5, a FAD-dependent oxidoreductase, might be involved with single demethylation to an oxidative process linked to CTB9 and CTB10 mediated methylenedioxybridge formation (Chapter 6) (60). However, it is currently unknown whether this step is mediated by CTB5 or CTB7 (Chapter 6) (Fig.5). *C. nicotianae* mutants lacking *CTB5* were reported to secret a dark orange/red monomeric quinone intermediate which was named cercosquinone B. Cercoquinone B is likely an oxidized and therefore stable form of the true naphthalene intermediate which serves as substrate for CTB5. Furthermore, loss of *CTB5* functionality resulted in reduced virulence (67). Homologs of this enzyme can also be found in the elsinochrome, phleichrome, and hypocrellin clusters (Chapter 6) (Fig. 3).

CTB6 is a NADPH-dependent oxidoreductase and thought to be the enzyme processing the intermediate formed by CTB2 (Chapter 6) (60) (Fig. 5). Its function is hypothesized to be the stereospecific installment of side chain hydroxyl groups. Inoculations assays revealed that *CTB6* disruption mutants of *C. nicotianae* cause less symptoms compared to the wild type (67). Interestingly, a truncated homolog of this enzyme can be found in the putative phleichrome pathway while no homolog is present in the elsinochrome and hypocrellin pathways (Fig. 3).

CTB7 is a 450 amino acid FAD-binding monooxygenase that harbors two FMN/FAD-binding domains and an amidation site (68). Interestingly, *CTB7* is present in a truncated form compared to *CTB7* of the CTB clusters of other *Cercospora* species in the cercosporin biosynthesis pathway of the grey leaf spot pathogen *C. zeina*. *C. zeina* with its naturally truncated and therefore non-functional *CTB7* as well as *CTB7* disruption mutants of *C. nicotianae* are deficient in cercosporin production *in vitro* (60, 68). It was proposed that this enzyme (or CTB5) is involved in the priming of the cercosporin precursor for methylenedioxy bridge installment, likely by removing one aryl methoxy group from the precursor (60) (Fig. 5). Interestingly, this function was already attributed before it was discovered that CTB cluster consists of more than eight genes (Chapter 5). Furthermore, except for the hypocrellin cluster also the elsinochrome and phleichrome cluster harbor this gene, despite that the products of these pathways lack the cercosporin specific methylenedioxy bridge in their structure (Chapter 6) (Fig. 3).

As a $Zn(II)Cys_6$ zinc finger transcription factor, **CTB8** is not directly involved in the modification of the toxin itself. However, it was shown to mediate CTB cluster gene expression, as *CTB8* disruption in *C. nicotianae* affected transcription of *CTB1* through *CTB7* (64). The lack of *CTB8* regulation also led to severe reduction of *CTB1* to *CTB7* gene expression levels and consequently to abolishment of cercosporin production *in vitro* and reduced virulence *in planta*. While *CTB8* is not only tightly incorporated in the CTB cluster in *C. beticola*, gene homologs are also present in the phleichrome and hypocrellin cluster (Chapter 6) (Fig. 3). However, it seems that the elsinochrome cluster in *E. fawcettii* harbors a truncated homolog of *CTB8* whose functionality has not been studied yet.

CFP (cercosporin facilitator protein) is another MFS transporter which is tightly incorporated in the CTB cluster of *C. beticola* (Chapter 5) (69). This transporter is hypothesized to partially provide toxin tolerance to the perylenequinone cercosporin via toxin export (see below: cercosporin auto-resistance) (53). Unlike the other MFS transporter CTB4, CFP homologs can also be found in the predicted phleichrome, elsinochrome, and hypocrellin biosynthetic clusters suggesting a conserved function in perylenequinone auto-resistance (Chapter 6) (Fig. 3).

CTB9 and **CTB10** are the only two genes without homologs in any known perylenequinone synthetic clusters (Chapter 6) (Fig. 3). While *CTB9* is predicted to be an α-ketoglutarate-depended dioxygenase, *CTB10* is suggested to encode a putative dehydratase (Chapter 5). Together they have been shown to be responsible for methylenedioxy bridge formation which is exclusively found in the cercosporin molecule. It is hypothesized that after CTB5- or CTB7-mediated precursor priming, CTB9 induces oxidative cyclization of the peculiar seven-membered ring while CTB10 possibly facilitates this reaction since *C. beticola CTB9* and *CTB10* deletion mutants were both shown to secret the corresponding precursor of the final cercosporin molecule (Fig. 5).

CTB11 is predicted as a βig-h3 fasciclin that together with the as a laccase annotated **CTB12** are hypothesized to be responsible for cercosporin dimerization, a process likely occurring early in the biosynthetic pathway (Chapter 5) (Fig. 5). Contribution to biosynthesis has been displayed when *C. beticola* mutants lacking either *CTB11* or *CTB12* were unable to produce cercosporin (Chapter 5). Both enzymes have homologs in elsinochrome, phleichrome and hypocrellin biosynthesis pathways (Chapter 6) (Fig. 3).

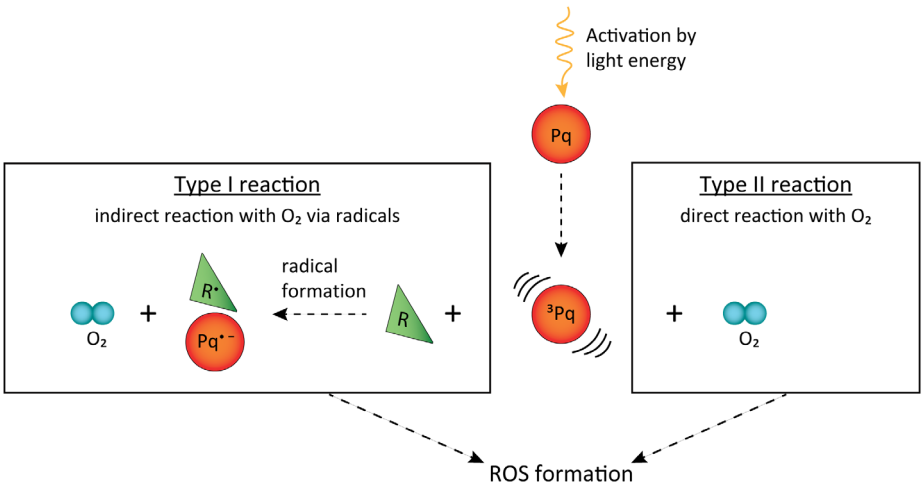

Figure 6. Perylenequinone mode-of-action. Light exposure activates perylenequinones (Pq) to reach an energetically excited triplet state (^{3}Pq) in which they can react with oxygen (O_2) to form reactive oxygen species (ROS). This reaction can happen indirectly (type I reaction) where the activated perylenequinone reacts with a reducing substrate (R) first resulting in radical formation (R• and Pq• -) which can react with O_2 to form ROS. Alternatively, the activated perylenequinone can react directly with O_2 (type II reaction) to form ROS.

Interestingly, the elsinochrome biosynthesis pathway in *E. fawcettii* and the hypocrellin pathway in *S. bambusicola* have an additional gene, annotated as a salicylate hydroxylase that has no homologs in the cercosporin or phleichrome pathway (Chapter 5) (Fig. 3). While the exact function of this pathway gene is yet to be determined, other fungal salicylate hydroxylases have been reported to be involved in naphthalene break-down (70, 71) and resistance provision in *Aspergillus nidulans* against the antifungal agent terbinafine (72).

Perylenequinone/cercosporin mode-of-action

The photosensitizing nature of various perylenequinones such as cercosporin, elsinochromes and fagopyrin has been known for a long time (73-75). The essential common structural feature responsible for photodynamic activity as well as color of the molecule is the 3,10-dihydroxy-4,9-perylenequinone chromophore (54). This core structure allows absorption of visible and near-UV light whereby the perylenequinones reach an electronically excited triplet state (76, 77). Once in this activated triplet state, two types of reactions can follow (76, 78, 79) (Fig. 6).

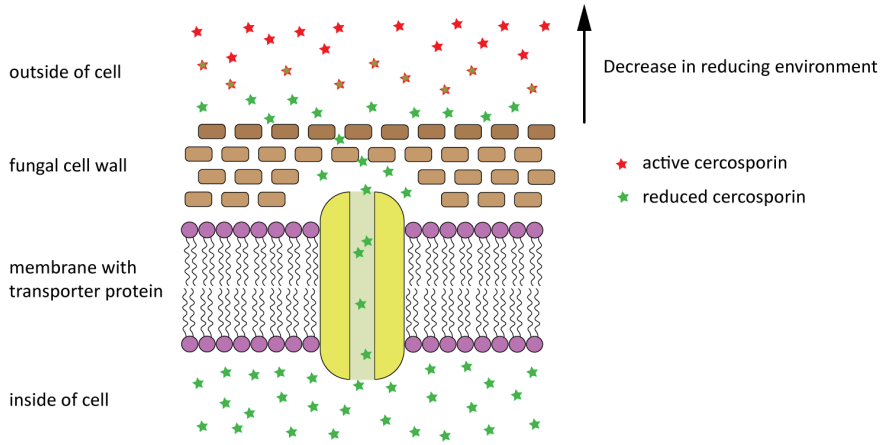

Figure 7. Auto-resistance mechanisms against perylenequinones. Perylenequinone-producing fungi have been shown to protect themselves by exporting the toxic compound outside of the cell via transporter proteins and by detoxification of the produced perylenequinone by creating an environment in which the toxin is getting reduced and therefore less toxic.

The excited perylenequinone can react with oxygen either indirectly (Type I reaction) through a reducing substrate or directly (Type II reaction). Interaction with an electron donor leads to the formation of free radicals or radical ions that upon reaction with oxygen produce reactive oxygen species (ROS) such as H_2O_2 and the free radical forms $O_2^{\bullet -}$, $HO_2^{\bullet}$, $OH^{\bullet}$. In a direct interaction between a triplet perylenequinone and oxygen, energy can be transferred from the excited triplet state perylenequinone to oxygen resulting in an excited singlet state of oxygen, also known as "singlet oxygen" ($^1O_2$). Both type I and II reactions yield highly ROS that at high concentrations are harmful to cells as they can cause lipid peroxidation, and protein and DNA damage (80, 81).

Cercosporin auto-resistance

It is essential for fungi that produce SMs with anti-fungal activity to be able to protect themselves from their own toxin. While genes involved in toxin biosynthesis usually are clustered and form a biosynthesis cluster specific for each secondary metabolite, these clusters quite often also harbor genes that seem to have no direct involvement in toxin production. Nevertheless, these additional genes are conserved within the cluster, suggesting a potential role in toxin tolerance. Keller (2015) (82) highlighted three self-resistance strategies, namely toxin export, detoxification, and duplication of the toxin target, known to date that originate from genes that are incorporated in toxin biosynthetic clusters. While the strategy of duplicating or creating a resistant target deployed by many antifungal toxin producing fungi has not been reported in the context of perylenequinone resistance, toxin export via transporter proteins and detoxification of toxic compounds have been found to be resistance mechanisms utilized by cercosporin-producing fungi (Fig. 7).

Transporter proteins

Protection by toxin efflux relies on the transportation of toxic substances from the inside of a cell to the outside through membrane transport proteins (Fig. 7). Transporters involved in self-resistance mainly belong to the major facilitator superfamily (MFS) and the ABC superfamily (83, 84). Both super families are often associated with multidrug resistance. An example of a transporter utilized by perylenequinone producing fungi is the MFS transporter CFP (53). *CFP* was first identified in *C. kikuchii* and shown to be involved in cercosporin auto-resistance. Targeted gene disruption of *CFP* in *C. kikuchii* resulted in mutant strains that displayed increased susceptibility to exogenous cercosporin, as their growth on cercosporin-amended media compared to media lacking additional cercosporin was reduced. Additionally, expression of *CFP* in the cercosporin-sensitive fungus *Cochliobolus heterostrophus* led to an increased toxin tolerance (85). Furthermore, it was shown that *CFP* disruption mutants produce much less cercosporin than the WT strain when grown in liquid culture under light conditions, indicating that *CFP* is also a determining factor for cercosporin production (53), possibly as a self-preservation effect. Studies in *C. nicotianae* identified three transporters; a CFP homolog and two ABC transporters ATR1 and ATR2 that play a role in cercosporin resistance (86). While gene disruption of *CFP* or *ATR1* resulted in increased sensitivity of the fungus to cercosporin (66, 86), overexpression of *ATR2* in the cercosporin sensitive fungus *Neurospora crassa* significantly increased its toxin tolerance (87). However, *ATR2* disruption mutants in *C. nicotianae* were not altered in toxin sensitivity but gene expression analysis revealed that the loss of ATR2 function led to an increased *CFP* expression. Partial functional compensation was also observed for CFP and ATR1 as constitutive overexpression of *ATR1* in *CFP* disruption mutants partially restored fungal tolerance to cercosporin (86). The potential to provide some level of tolerance against perylenequinones was also observed for ABC and MFS transporter family members identified in non-perylenequinone producing organisms. Native MFS and ABS transporters in *Botrytis* cinerea and the yeast *Saccharomyces cerevisiae*, respectively, have been reported to confer cercosporin resistance when overexpressed (88, 89). Interestingly, it was possible to transfer this transporter-based resistance mechanism to plants. For example, stable transformation of fungal-derived *CFP* in tobacco resulted in CFP+ transgenic plants with increased tolerance to cercosporin since application of external cercosporin on the leaves as well as infection with *C. nicotianae* resulted in smaller necrotic lesion sizes (90). With the discovery

that the CTB cluster consists of more genes than previously identified eight CTB genes, it was possible to demonstrate that the MFS transporter homolog in *C. beticola CbCFP*, also involved in auto-resistance, actually lies within the CTB cluster (Chapter 5) (69). In fact, it is tightly incorporated in the cluster, flanked from both sides by genes necessary for cercosporin production (Fig. 3). Its location within the cluster illustrates the close connection between toxin biosynthesis and auto-resistance and ensures conservation of toxin tolerance together with the mycotoxin production pathway as loss of self-resistance would be devastating for the fungus.

Detoxification by alteration of the toxin structure

Besides toxin export, the active modification of a toxin into a less toxic derivate is another self-protection strategy used by many fungi (82). Instead of depending on only one resistance mechanism, the auto-resistance repertoire of *Cercospora* spp. also includes the ability to defend against cercosporin by reductive detoxification of the cercosporin molecules (91-94). Although reduced cercosporin is rather labile and readily re-oxidizes upon removal of reducing agents or through air exposure (94, 95), analysis of stable methylated and acetylated reduced cercosporin derivatives revealed that they absorb less light and generate significantly less singlet oxygen ($^1O_2$) compared to wild-type cercosporin (94). Consequently, their potential to cause lipid peroxidation *in vitro* was highly reduced and cercosporin-susceptible fungi grew significantly bigger on media amended with reduced cercosporin compared to wild-type cercosporin containing media (94). Furthermore, it was found that $^1O_2$ production by reduced cercosporin is highly influenced by its chemical environment with a distinct low in an aqueous setting as it can be found inside of cells (91). Through fluorescence microscopy with specific band-width filters, it was possible to discriminate reduced cercosporin from wild-type cercosporin (92). Interestingly, when grown in the presence of wild-type cercosporin, hyphae of *Cercospora* species as well as a cercosporin-resistant *Alternaria alternata* strain were not emitting red wild-type cercosporin-specific fluorescence but green fluorescence associated with reduced form of cercosporin. The reduced cercosporin was later found to be localized in the cytoplasm of the fungal cells (91). Non-viable *Cercospora* spp. and cercosporin-sensitive *N. crassa* and *Aspergillus flavus* strains on the other hand were unable or highly limited in their abilities to reduce cercosporin and therefore emitted red wild-type cercosporin specific fluorescence (92). Further investigations revealed that the cell surface of cercosporin-resistant strains is surrounded by a reducing environment (94). Thereby resistant fungi are able to reduce nearby cercosporin into its less reactive form and keep it in this state as long as it is in close proximity to the fungal hyphae (Fig. 7). The importance of reduction as a defense mechanism for oxidative stress tolerance was supported by the discovery of the transmembrane reductase *Cpd1* (cercosporin and photosensitizer detoxification) in yeast (89). Mutants over-expressing *Cpd1* showed an increased resistance to cercosporin and other synthetic photosensitizers whereas disruption of the gene lead to increased susceptibility. Furthermore, Cpd1 is able to increase cercosporin tolerance of plants as leaves of transgenic tobacco plants expressing *Cdp1* were less affected by infiltration of pure cercosporin (96). Although the exact functional mechanism of Cpd1 is still unknown, *Cpd1* shows significant similarities FAD-dependent pyridine nucleotide reductases suggesting a possible role in reduction of the cercosporin molecule. Although it is reported for other fungi that genes linked to active toxin detoxification can be found incorporated in the biosynthesis cluster, no CTB genes have been affiliated with auto-resistance by cercosporin reduction yet.

Other resistance mechanisms

While toxin efflux and detoxification have been shown to be resistance strategies of perylenequinone-producing fungi, studies on self-resistance also led to the identification of other resistance mechanisms and genes that play a role in toxin tolerance but for which the underlying mechanisms are not yet fully understood. Interestingly, a gene involved in vitamin B_6 (pyridoxine) biosynthesis is also among the additionally identified resistance-providing genes (97). SOR1 (Singlet Oxygen Resistance 1), later renamed to PDX1 based on its pyridoxine auxotrophy phenotype, was identified due to its ability to restore cercosporin resistance in an UV-derived toxin-sensitive mutant (98, 99). Targeted gene replacement of *PDX1* resulted in increased susceptibility to cercosporin and other photosensitizers. Since further research revealed that PDX1 is required for vitamin B_6 formation, the role of pyridoxine and its derivatives in protection against photosensitizers was investigated further (97, 100). Pyridoxine, pyridoxal, pyridoxal 5-phosphate, and pyridoxamine were all found to be potent $^1O_2$ quencher and therefore are likely contributing to oxidative stress resistance caused by cercosporin and other photosensitizers.

Another example is the transcription factor *Crg1* (cercosporin resistance gene) identified in *C. nicotianae* (101, 102). Crg1 was found to be partially involved in cercosporin auto-resistance as *C. nicotianae Crg1* disruption mutants display significantly impaired growth on cercosporin-amended media. However, growth of *Crg1* disruption mutants was not significantly altered by the presence of other $^1O_2$ generating photosensitizers (eosin Y and toluidine blue). This finding indicates that CRG1 seems to regulate specific genes involved in cercosporin tolerance rather than genes important for $^1O_2$ resistance in general.

Conclusion

While knowledge on the effector biology of the hemibiotrophic sugar beet pathogen *C. beticola* is limited, research aiming to identify and characterize new effectors is viable to understand the underlying molecular strategies that this fungus employs to establish disease. The currently known effector repertoire of *C. beticola* indicates that this fungus utilizes proteinaceous as well as SM effectors during infection. Furthermore, it appears that during its necrotrophic phase, *C. beticola* is equipped with specific necrosis-inducing effectors for light (cercosporin) and dark periods (CbNip1). The broad toxicity of the perylenequinone cercosporin makes it dangerous not only to plants but potentially also to other living organisms. With the discovery that the ability to synthesize cercosporin is not limited to the *Cercospora* genus a new level of complexity on perylenequinones as potential health threats emerged since perylenequinone-producing pathogens may secrete these toxins into plant products that are directly consumed by mammals. The increased use of next-generation sequencing combined with bioinformatics and molecular-biological approaches can help to uncover latent perylenequinone producers and will shed light on yet unexplored areas of perylenequinone research. To date, our knowledge on underlying auto-resistance mechanisms is still limited. However new insights in this field will help to increase our understanding of how toxin tolerance is achieved by fungi and can potentially lead to new developments for modern agricultural farming.

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Summary

Plants possess an innate immune system that enables them to detect microbial invasion and respond accordingly to prevent disease. In turn, microbes have evolved secreted molecules that are collectively termed effectors to overcome recognition by the plant and facilitate host colonization. In **Chapter 1**, past and current conceptual models depicting plant-microbe interplays during infection are addressed. Additionally, the primary subject of this thesis, the sugar beet pathogen *Cercospora beticola*, is introduced.

Depending on their life style, plant pathogenic fungi employ versatile virulence strategies during colonization. **Chapter 2** provides a broad overview of virulence mechanisms that are utilized by various pathogenic microbes. Furthermore, it highlights different kinds of effectors such as proteins, small RNAs, and secondary metabolites, and their function in context of fungal virulence.

An example of a proteinaceous effector in *C. beticola* is CbAve1, a homolog of the *Verticillium dahliae* VdAve1 (*Verticillium dahliae* Avirulence on Ve1 tomato) effector. In *V. dahliae*, this effector is exclusively found in race 1 strains and has been shown to be an avirulence protein as recognition by the tomato Ve1 receptor leads to resistance against *V. dahliae* strains harboring VdAve1. Besides in *C. beticola* (*CbAve1*), homologs of *VdAve1* are found in some plant pathogenic fungi such as *Fusarium oxysporum* (*FoAve1*) and *Colletotrichum higginsianum* (*ChAve1*) as well as in many plant species. Since VdAve1 has been reported to be a virulence factor for *V. dahliae*, **Chapter 3** investigates whether the homologs that have been found in *C. beticola*, *F. oxysporum*, and *Co. higginsianum* also contribute to virulence of their producers. While *CbAve1* and *FoAve1* are shown to be expressed during the fungal infection process and contribute to virulence of their producer, site-directed deletion of *ChAve1* in *Co. higginsianum* had no effect on fungal virulence and no expression was detected during *Co. higginsianum* on Arabidopsis. Interestingly, earlier studies demonstrated that none of the fungal homologs of VdAve1 were able to complement Δ*VdAve1 V. dahliae* mutants to full virulence. Thus, it is hypothesized that VdAve1 functions differently from the other fungal homologs.

As little is known about the proteinaceous effector repertoire of *C. beticola*, we aimed to identify novel effector proteins. Since *C. beticola* is a hemibiotrophic fungus, it relies on host cell death induction during the necrotrophic stage of its lifecycle. **Chapter 4** describes a phenotype-based approach to identify novel effector proteins that can evoke host cell death. Growing *C. beticola* in Fries medium for seven days resulted in fungal culture filtrate that led to necrosis formation in sugar beet leaves upon infiltration. Mass spectrometry analysis of necrosis-inducing culture filtrate fractions yielded three effector candidates. While two candidates were excluded from further studies due to the lack of effector characteristics or the absence of a necrosis-inducing function, the effector candidate CbNip1 fulfilled all criteria and was further analyzed. The novel necrosis-inducing protein CbNip1 is a small, secreted, cysteine-rich effector protein that is expressed during infection and contributes to *C. beticola* virulence by inducing necrosis in plant leaves in the absence of light.

C. beticola is well-known to utilize the perylenequinone cercosporin for necrosis induction during infection. Decades of research on the cercosporin toxin biosynthetic (CTB) pathway identified eight genes that contribute to toxin production. In **Chapter 5**, phylogenomic analysis revealed that the CTB cluster is larger than previously reported and includes five additional genes located at the 3' end of the original CTB gene cluster in *C. beticola* that are essential for toxin production. Among those five genes, two were identified that are responsible for the formation of the methylenedioxy bridge; a unique feature of the cercosporin molecule that is not found in other perylenequinones characterized to date. Furthermore, phylogenomic analysis unveiled that the CTB cluster underwent duplication and horizontal transfer events and is therefore present in a diverse range of plant pathogenic fungi, of which *Colletotrichum fioriniae* was shown to have the ability to produce cercosporin. Furthermore, gene expression analysis of the *Co. fioriniae* CTB cluster PKS gene *CofCTB1* confirmed that cercosporin production is activated during infection.

The cercosporin molecule displays high structural similarity to other perylenequinone family members such as elsinochrome and phleichrome synthesized by fungi *Elsinoë fawcettii* and Cladosporium phlei, respectively. Therefore it was hypothesized in **Chapter 6** that the biosynthetic gene clusters responsible for production of these perylenequinones are likely to exhibit significant similarity. Based on gene cluster conservation, it was revealed that earlier efforts to identify elsinochrome and phleichrome pathways resulted in misidentification and the biosynthetic pathways claimed to be responsible for their production are actually involved in DHN-melanin biosynthesis. Furthermore, due to the overall gene conservation within perylenequinone biosynthetic clusters, it was possible to identify the true perylenequinone pathways for elsinochrome in *E. fawcettti* and phleichrome in *C. phlei* using the established CTB cluster as reference.

Aiming to shed further light on the effector repertoire of *C. beticola*, this thesis investigated proteinaceous and secondary metabolite effectors of this fungus. **Chapter 7** provides an overview of currently known *C. beticola* effectors and associated mechanisms.

Acknowledgements

A clear advantage of doing a "sandwich" PhD is that you get to make friends at two different locations. There have been so many wonderful people who have contributed to this thesis and who have made my life so much more colorful and fun. Thank you all for your help and for making this journey so special for me!

First of all, I feel incredibly fortunate to have the two coolest people in the world as promoters. Without Bart and Melvin, this thesis would simply not have been possible. Thank you both for creating a positive and enthusiastic working environment, it made occasional set-backs much easier to deal with. Watching you guys has also shown me that it is perfectly possible to be honest and kind and still be successful in science. I also really appreciate that you not only guided me through the PhD but you also prepared me for the next step and I am now looking forward to finding my place in science.

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Melvin, I know it is getting old because I have been saying this for the last 4 ½ years but I am still convinced that coming to Fargo was without a doubt the BEST decision I have ever made in my life! From day one you had complete trust in my skills and fully supported me in what I did and I cannot thank you enough for that! You are not only my promotor but you became a really good friend and I know that I can rely on you 100%. Thanks! And an especially big 'Thank you!' also goes to your family. Starting with Tonya, Ava and Wren for always being awesome, helpful and fun to hang out with and also to your parents and your siblings and their families, and all the other Boltons and Beckers who I got to meet, for making me feel welcome at all times. You and your whole family were all so kind to me and made my stay in Fargo incredibly special! Thanks!!

In theory, this is the part where I was planning on transitioning to thanking everyone chronologically starting with my friends and colleagues from Fargo and followed by the ones from Wageningen but there is one person who stands out and who I can't seem to fit into this scheme. Ronnie, without you this thesis would not have been possible! Thank you so much for answering all my questions with incredible patience, for involving me in projects and also for all the discussions about science and 'real life' that we had.

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About the author

Malaika K. Ebert was born in Frankfurt a. M. (Germany) on May 8th, 1985. In 2007, she started her BSc in Agricultural Science at the University of Bonn and wrote her BSc thesis on 'The effect of *Venturia inaequalis* on chlorophyll content, photosynthetic efficiency and fluorescence of apple leafs' under the supervision of Dr. Ulrike Steiner.

In 2010, Malaika started an MSc in Plant Science with a specialization in Plant Pathology and Entomology at Wageningen University. For her first MSc thesis, she joined the group of Prof. Dr. Bart Thomma at the Laboratory of Phytopathology and, under supervision of Dr. Anja Kombrink, worked on the project titled 'The influence of LysM effectors in fungal virulence and in interactions with other microorganisms.' Since she also wanted to learn about the interaction of plants with beneficial microbes, Malaika joined the group of Dr.ir. René Geurts at the Laboratory of Molecular Biology to perform her second MSc thesis. Under the supervision of Arjan van Zeijl, she identified and characterized ACC synthase genes in *Parasponia* and *Trema*.

In 2014, Malaika was appointed as a Sandwich PhD under the joined supervision of Prof. Dr. Bart Thomma (WUR) and Dr. Melvin Bolton (USDA, NDSU) to unravel the effector biology of the sugar beet pathogen *Cercospora beticola*. After an initial two years at the laboratory of Dr. Melvin Bolton at the U.S. Dept. of Agriculture and North Dakota State University, Malaika returned to the team of Prof. Dr. Bart Thomma for the second half of her PhD. Later that year, Malaika was awarded the EPSO Young Plant Scientist award 2016 in applied research for the suggestion to engineer durable disease resistance by transferring fungal toxin auto-resistance genes to plants. Since July 2018, Malaika is a postdoctoral researcher in the group of Prof. Dr. Gunther Doehlemann at the University of Cologne where she investigates the effector repertoire of the smut fungus *Ustilago maydis*.

de Jonge R.*,#, **Ebert M.K***, Huitt-Roehl C.R.*, Pal P., Suttle J.C., Neubauer J.D., Jurick W.M., Secor G.A., Thomma B.P.H.J., Van de Peer Y., Townsend C.A.#, Bolton M.D.# (2018) Gene cluster conservation provides insight into cercosporin biosynthesis and extends production to the genus *Colletotrichum*. Proceedings of the National Academy of Sciences. DOI: 10.1073/pnas.1712798115
*,# The contribution of these authors should be considered equal.

Rodriguez-Moreno L.*, **Ebert M.K.***, Bolton M.D.#, Thomma B.P.H.J.# (2018) Tools of the crook – infection strategies of fungal plant pathogens. The Plant Journal. DOI: 10.1111/tpj.13810
*,# The contribution of these authors should be considered equal.

Bolton MD, **Ebert M.K.***, Faino L*, Rivera-Varas V, de Jonge R, van de Peer Y, Thomma BPHJ, Secor G (2016). RNA-Sequencing of *Cercospora beticola* DMI-sensitive and -resistant isolates after treatment with tetraconazole identifies common and contrasting pathway induction. Fungal Genetics and Biology. DOI: https://doi.org/10.1016/j.fgb.2016.04.003
* The contribution of these authors should be considered equal.

Ebert M.K.*, Spanner R.E.*, de Jonge R.*,#, Smith D.J., Holthusen J., Secor G.A.,Thomma B.P.H.J., Bolton M.D#. Gene cluster conservation identifies melanin and perylenequinone biosynthesis pathways in multiple plant pathogenic fungi. Submitted.
*,# The contribution of these authors should be considered equal.

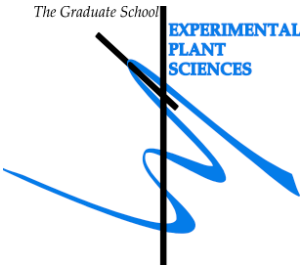
Ebert M.K., Wang X., Friesen T.L., de Jonge R., Neubauer J.D., Secor G.A., Thomma B.P.H.J., Bolton M.D. Identification and characterization of *Cercospora beticola* necrosis-inducing effector CbNip1, Submitted.

Boshoven J.C., **Ebert M.K.***, Song Y.*, Rovenich H., Rojas Padilla E., Bolton M. D., Thomma B.P.H.J. Homologs of *Verticillium dahliae* effector Ave1 contribute to virulence of various fungal pathogens. Submitted.
* The contribution of these authors should be considered equal.

Ebert M.K., Thomma B.P.H.J., Bolton M.D. Perylenequinones in plant pathology. In preparation.

De Jonge R., Bian Z., Webb K.M., **Ebert M.K.**, Spanner R.E., Shrestha S., Bolton M.D. Identification and characterization of *Fusarium secorum* effector proteins. Author order is not fixed. In preparation.

Education Statement of the Graduate School Experimental Plant Sciences



Issued to: **Malaika Karolina Ebert**
Date: **6 September 2018**
Group: **Laboratory of Phytopathology**
University: **Wageningen University & Research**

1) Start-up phase		*date*
➠	**First presentation of your project**	
	Title: Disease management and effector biology of the sugar beet pathogen Cercospora beticola	02 Feb 2014
➠	**Writing or rewriting a project proposal**	
	Title: Disease management and effector biology of the sugar beet pathogen Cercospora beticola	11 Mar 2013
➠	**Writing a review or book chapter**	
	Tools of the crook – infection strategies of fungal plant pathogens, submitted to The Plant Journal	15 Nov 2017
➠	**MSc courses**	
➠	**Laboratory use of isotopes**	
	Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure		*date*
➠	**EPS PhD student days**	
	EPS PhD Student days 'Get2Gether', Soest, NL	09-10 Feb 2017
	EPS PhD Student days 'Get2Gether', Soest, NL	15-16 Feb 2018
➠	**EPS theme symposia**	
	EPS Theme 2 symposium ' Interactions between Plants and Biotic Agents', together with Willie Commelin Scholten day, Leiden, NL	22 Jan 2016
	EPS Theme 2 symposium ' Interactions between Plants and Biotic Agents', together with Willie Commelin Scholten day, Wageningen, NL	23 Jan 2017
	EPS Theme 3 symposium "Metabolism and Adaptation', Wageningen, NL	14 Mar 2017
➠	**National meetings (e.g. Lunteren days) and other national platforms**	
	Annual meeting 'Experimental Plant Sciences', Lunteren, NL	11-12 Apr 2016
	Annual meeting 'Experimental Plant Sciences', Lunteren, NL	10-11 Apr 2017
➠	**Seminars (series), workshops and symposia**	
	Seminars:	
	Prof.dr. Jane Parker, Resistance pathway dynamics in plant immunity, Wageningen	21 Jan 2016
	Prof. Laura Grenville-Briggs 'Molecular Oomycete-Host Interactions: The Good, the Bad and the ugly', Wageningen	19 Feb 2016

	Jan Ruijter "Analysis of qPCR data. The use and usefulness of amplification curve analysis", Wageningen	14 Mar 2016
	Dr. Olivier Hamant 'How do plants read their own shape ?', Wageningen	16 Mar 2016
	Prof. Alain Tissier 'Insights into the inner workings of a metabolic cell factory: the tomato glandular trichome'	18 Mar 2016
	Prof.dr. Douglas Mitchell 'Genomics-enabled natural products discovery', Wageningen	31 Mar 2016
	Prof. Caitilyn Allen, 'How Ralstonia solanacearum succeeds in plant xylem vessels', Wageningen	29 Apr 2016
	Workshops:	
	COST Israel,Tel Aviv, Israel	10-12 Feb 2015
	Metabolomics in Chemical Ecology, NIOO, Wageningen, NL	31 Oct-01 Nov 2016
	COST annual meeting, Bled, Slovenia	01-03 Mar 2017
	Symposia:	
	3rd Wageningen PhD Symposium "Diversity of Science" - entitled Learn from your enemy – transferring a pathogen-derived toxin resistance mechanism to crop plants for durable disease resistance	26 Apr 2016
▶▶	**Seminar plus**	
▶▶	**International symposia and congresses**	
	APS annual meeting Minneapolis, MN, USA	09-13 Aug 2014
	Asilomar Fungal Genetics, Pacific Grove, CA, USA	17-22 Mar 2015
	ASSBT - American Society of Sugar Beet Technologists biennial meeting, Clearwater Beach, FL, USA	23-26 Feb 2015
	EPSO Plant Biology Europe, Prague, CZE	26-30 Jun 2016
▶▶	**Presentations**	
	Talks:	
	APS "Characterization of novel Cercospora beticola effector proteins " Minneapolis, USA	11 Aug 2014
	ASSBT "The Cercospora beticola effector CbAve1 promotes virulence during sugar beet infection", Clearwater Beach, USA	24 Feb 2015
	COST "Identification and characterization of a novel Cercospora beticola effector protein", Tel Aviv, Israel	12 Feb 2015
	Lunteren "Identification of fungal toxin auto-resistance genes and their potential to provide durable resistance in crop plants", Lunteren, NL	11-12 Apr 2016
	3rd Wageningen PhD Symposium "Diversity of Science" - entitled Learn from your enemy – transferring a pathogen-derived toxin resistance mechanism to crop	26 Apr 2016
	Molecular Genetics meeting "Fungal toxin self-resistance genes and their potential to provide durable resistance in crops", Wageningen, NL	21 Oct 2016
	COST Annual meeting "Transfer of fungal-derived toxin tolerance to crop plants to engineer resistance to Cercospora diseases" Bled, Slovenia	01-03 Mar 2017
	Poster:	
	Fungal genetics conference "Characterization of the novel Cercospora beticola necrosis-inducing effector CbNIP10", Pacific Grove, USA	17-22 Mar 2015
▶▶	**IAB interview**	
▶▶	**Excursions**	
	Subtotal Scientific Exposure	*20.7 credits**

3) In-Depth Studies		*date*
➠	**EPS courses or other PhD courses**	
	Your Plant Science NIAB PhD Winter School, UK	20-21 Nov 2017
	Advanced course 'Data analysis and visualizations in R (for biologists)', Wageningen, NL	12-13 Dec 2016
➠	**Journal club**	
➠	**Individual research training**	
	FARGO, Dr Melvin Bolton, sugar beet unit, United States Department of Agriculture, Fargo, USA	02 Feb 2014-31 Dec 2015
	Subtotal In-Depth Studies	*4.2 credits**

4) Personal development		date
➠	**Skill training courses**	
	EPS Introduction Course, Wageningen, NL	11 Feb 2017
	Reviewing a scientific paper, Wageningen, NL	17 Mar 2016
	Writing grant proposals, Wageningen, NL	09 Sep-22 Nov 2016
➠	**Organisation of PhD students day, course or conference**	
	Get2Gether - pubquiz	09-10 Feb 2017
	Flying seminars (Mak Varrelmann, 18 Apr 2017; Urs Wyss, 2 Oct 2017)	2016 and half of 2017
➠	**Membership of Board, Committee or PhD council**	
	PhD council	2016 and half of 2017
	Subtotal Personal Development	*4.6 credits**

TOTAL NUMBER OF CREDIT POINTS*	**43,0**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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