

1 Ancient duplication and horizontal transfer of a toxin gene cluster reveals novel mechanisms in the  
2 cercosporin biosynthesis pathway

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1 **Abstract**

2 *Cercospora* species have a global distribution and are best known as the causal agents of leaf spot diseases  
3 of many crops. *Cercospora* leaf spot (CLS) is an economically devastating disease of sugar beet caused by  
4 *C. beticola*. The *C. beticola* genome encodes 63 biosynthetic gene clusters, including the cercosporin toxin  
5 biosynthesis (*CTB*) cluster. Studies spanning nearly 60 years have shown that cercosporin is photo-  
6 activated, critical for disease development, and toxic to most organisms except *Cercospora* spp.  
7 themselves, which exhibit cercosporin auto-resistance. We show that the *CTB* gene cluster has  
8 experienced an unprecedented number of duplications, losses, and horizontal transfers across a spectrum  
9 of plant pathogenic fungi. Although cercosporin biosynthesis has been widely assumed to rely on the eight  
10 gene *CTB* cluster, our comparative genomic analysis revealed extensive gene collinearity adjacent to the  
11 established cluster in all *CTB* cluster-harboring species. We demonstrate that the *CTB* cluster is larger than  
12 previously recognized and includes the extracellular proteins fasciclin and laccase required for cercosporin  
13 biosynthesis and the final pathway enzyme that installs the unusual cercosporin methylenedioxy bridge.  
14 Additionally, the expanded cluster contains CFP, which contributes to cercosporin auto-resistance in *C.*  
15 *beticola*. Together, our results give new insight on the intricate evolution of the *CTB* cluster.

1 *Cercospora* are among the most speciose genera in all Fungi. First described in 1863<sup>1</sup>, the genus has  
2 sustained a long history largely due to notoriety as the causal agent of leaf spot diseases in a wide range  
3 of plants including agriculturally important crops such as sugar beet, soybean, maize and rice that together  
4 account for hundreds of millions of dollars in lost revenue annually to growers worldwide. Although  
5 *Cercospora* spp. share a number of characteristics associated with pathogenicity, including penetration  
6 through natural openings and extracellular growth during the biotrophic stage of infection, most rely on  
7 the production of the secondary metabolite (SM) cercosporin to facilitate infection<sup>2,3</sup>. SMs are bioactive  
8 molecules that play crucial roles in the establishment of specific ecological niches but, unlike primary  
9 metabolites, are not essential for fungal growth, development or reproduction. Cercosporin is a  
10 perylenequinone that is nearly universally toxic to a wide array of organisms including bacteria, mammals,  
11 plants, and most fungal species with the key exception of cercosporin-producing fungi, which secrete  
12 cercosporin in millimolar quantities with no apparent effect on growth and, therefore, exhibit auto-  
13 resistance<sup>4</sup>. When exposed to ambient light, cercosporin is a potent producer of reactive oxygen species  
14 (ROS) in the presence of oxygen<sup>5</sup> with a quantum efficiency of >80%<sup>6</sup>. This small molecule is lipophilic and  
15 can readily penetrate plant leaves leading to indiscriminant cellular damage within seconds of exposure<sup>7</sup>.  
16 Therefore, the physiological and genetic mechanisms underlying the ability for *Cercospora* spp. to tolerate  
17 cercosporin and associated ROS is an intriguing area of research with potentially important practical  
18 applications.

19 In contrast to the large body of information on cercosporin biology spanning several decades<sup>8,9</sup>,  
20 the cercosporin toxin biosynthesis (*CTB*) gene cluster was only recently resolved in *C. nicotianae*<sup>10</sup>. The  
21 keystone enzyme for cercosporin biosynthesis, *CTB1*, bears all the hallmarks of an iterative, non-reducing  
22 polyketide synthase (NR-PKS)<sup>11</sup>. Using *CTB1* as a point of reference, the complete *C. nicotianae* *CTB* gene  
23 cluster was determined to consist of eight contiguous genes. Six of these genes are believed to be  
24 responsible for cercosporin assembly (*CTB1, 2, 3, 5, 6, and 7*)<sup>10,12</sup>. The zinc finger transcription factor *CTB8*

1 co-regulates expression of the cluster<sup>10</sup>, while the major facilitator superfamily (MFS) transporter CTB4  
2 exports the final metabolite<sup>13</sup>. Downstream of the *CTB* cluster are two open reading frames (ORFs)  
3 encoding truncated transcription factors while loci designated as *ORF9* and *ORF10* upstream of the *CTB*  
4 cluster are not regulated by light and are not hypothesized to encode proteins with metabolic functions<sup>10</sup>.  
5 Consequently, the clustering of eight genes with demonstrated co-regulation by light that are flanked by  
6 ORFs with no apparent role in cercosporin biosynthesis has suggested that cercosporin production relies  
7 on the eight-gene *CTB* cluster<sup>10</sup>. In this study, we used an evolutionary comparative genomics approach  
8 to show that the *CTB* gene cluster underwent multiple rounds of duplication and was transferred  
9 horizontally across large taxonomic distances. Since these horizontal transfer events included the transfer  
10 of genes adjacent to the canonical eight gene *CTB* cluster, we show that the *CTB* cluster includes additional  
11 genes in *C. beticola*, including at least one gene that contributes to cercosporin auto-resistance and three  
12 previously unrecognized genes involved with biosynthesis.

## 13 **Results**

### 14 **Secondary metabolite cluster expansion in *Cercospora beticola*.**

15 *C. beticola* strain 09-40 was sequenced to 100-fold coverage and scaffolded with optical and genome  
16 maps, resulting in 96.5% of the 37.06 Mbp assembly being placed in 12 supercontigs. Despite their  
17 ubiquitous presence in nature and in many cropping systems, genome sequences of *Cercospora* spp. are  
18 not well-represented in public databases. Therefore to aid comparative analysis within the *Cercospora*  
19 genus, we also sequenced the genome of *C. berteroae* and reassembled the genome of *C. canescens*<sup>14</sup>  
20 (Supplementary Table 1). To identify gene clusters responsible for biosynthesis of aromatic polyketides in  
21 *C. beticola*, we mined the genome to identify all SM clusters<sup>15</sup> and compared these with predicted clusters  
22 in related Dothideomycete fungi. The *C. beticola* genome possesses a total of 63 SM clusters of several  
23 classes (Supplementary Table 2), representing a greatly expanded SM repertoire compared to closely  
24 related Dothideomycetes (Supplementary Table 3). In order to identify the *C. beticola* PKS cluster

1 responsible for cercosporin production, we compared the sequence of the *C. nicotianae* *CTB* cluster<sup>10</sup> with  
2 predicted PKS clusters of *C. beticola*. The *C. beticola* PKS *CBET3\_00833* (*CbCTB1*) and flanking genes  
3 (*CBET3\_00830* – *CBET3\_00837*) were ~96% identical to *C. nicotianae* *CTB1* – *CTB8* and all genes were  
4 collinear, strongly suggesting this region houses the *CTB* cluster in *C. beticola*.

#### 5 **Repeated duplication and lateral transfer of the cercosporin biosynthetic cluster.**

6 To study the evolutionary relationships of *C. beticola* PKSs, we conducted large-scale phylogenomic  
7 analyses that included various previously characterized PKSs from selected species<sup>16</sup>. Since resolving  
8 orthologous relationships among PKSs can predict the type of SM that will be synthesized, we first built a  
9 phylogenetic tree of the conserved core ketosynthase (KS) domains of each PKS that resulted in separating  
10 PKS enzymes into four major groups (Supplementary Fig. 1). Among the eight *C. beticola* NR-PKSs,  
11 phylogenetic analysis revealed significant similarity between *CbCTB1* and *CBET3\_10910-RA*, which cluster  
12 at the base of the cercosporin clade (Supplementary Fig. 1). Interestingly, *CBET3\_10910* flanking genes  
13 were also strikingly similar to *CbCTB* cluster genes (Fig. 1). Consequently, we hypothesize that the  
14 *CBET3\_10910* SM cluster is the result of a *CTB* cluster duplication. Since duplicated SM gene clusters  
15 appeared to be relatively rare in fungi<sup>17</sup>, we investigated the origin and specificity of the *CTB* cluster and  
16 the putative duplication by searching for *CbCTB1* homologs against a selected set of 48 published  
17 Ascomycete proteomes (Supplementary Table 4) representing a diverse group of fungal orders. We  
18 identified *CbCTB1* orthologs in *Cercospora* spp. *C. berteroae* and *C. canescens* and confirmed its presence  
19 in *Cladosporium fulvum*<sup>16</sup> and *Parastagonospora nodorum*<sup>18</sup>. Surprisingly, seven additional orthologs were  
20 identified in Sordariomycete species *Colletotrichum orbiculare*, *C. gloeosporioides*, *C. fiorinae*, *C.*  
21 *graminicola*, *C. higginsianum*, and *Magnaporthe oryzae* as well as one in the Leotiomycete *Sclerotinia*  
22 *sclerotiorum* (Supplementary Fig. 2), representing a diverse taxa harboring *CTB1*. Analysis of sequence  
23 similarity showed that intra-species (*CbCTB1* - *CBET3\_10910-RA*) sequence similarity (65%) was lower than  
24 the inter-species similarity (e.g. *CbCTB1* and *C. fulvum* *CTB1* (Clafu1\_196875) are 73% similar;

1 Supplementary Table 5), suggesting that the *CTB1* duplication event was ancient and occurred prior to  
2 Dothideomycete speciation. Reconciliation<sup>19</sup> of the species tree with the *CTB1* protein tree revealed that  
3 the predicted evolutionary history of *CTB1* can be characterized by four duplications, three transfers and  
4 22 losses (Fig. 2), and further corroborate our hypothesis that the *CTB1* duplication event (D1) occurred  
5 prior to Dothideomycete speciation. Reconciliation revealed an ancient transfer in which the lineage  
6 leading to *S. sclerotiorum* acquired the duplicated *CTB1* from the last common ancestor of *Cercospora*  
7 spp. (T1; Fig. 2A, B). Besides *S. sclerotiorum*, the only species that has retained the duplicated *CTB1* gene  
8 are *Cercospora* spp. and *P. nodorum* (Fig. 2B). Duplications 2-4 (D2-4) arose after lateral transfer (T2) of  
9 *CTB1* into the last common ancestor of the *Glomerellales*. *CTB1* was then transferred (T3) from a common  
10 ancestor in the *Glomerellales* to *Magnaporthe oryzae* (Fig. 2).

11 We extended the search for CTB cluster protein orthologs by scanning the 48 proteomes for  
12 homologs of *CbCTB2* (CBET3\_00830) to *CbCTB8* (CBET3\_00837) followed by phylogenetic tree  
13 construction and subtree selection (Supplementary Fig. 3). This resulted in the identification of orthologs  
14 in the same set of species previously listed to contain *CTB1*, with the only exceptions in cases where *CTB*  
15 gene homologs were lost in a species. Reconciliation of the subtrees for *CTB2*, *CTB3*, *CTB4*, *CTB5* and *CTB8*  
16 (Supplementary Fig. 4) largely supported the proposed scenario for *CTB1* (Fig. 2B). Moreover, *CTB*  
17 orthologs were microsyntenic (Fig. 3). The extensive loss of *CTB6* and *CTB7* orthologs limits reconciliation  
18 analysis of these gene families. To further evaluate the significance of cross-species microsynteny, we  
19 estimated genome-wide microsynteny between *C. beticola* and *C. gloeosporioides* and between *C.*  
20 *beticola* and *M. oryzae* (Supplementary Fig. 5). Surprisingly, microsynteny of the *CTB* gene cluster is  
21 significantly differentiated from the genome-wide average, emphasizing its apparent evolutionary  
22 significance. *CTB* cluster GC content from *Cercospora* and *Colletotrichum* spp. was similar to the  
23 transcriptome-wide average in *Cercospora* spp. (54%) but different from the GC content of the  
24 *Colletotrichum* transcriptome (58%; Supplementary Table 6). Likewise, *CTB* protein similarity between *C.*

1 *beticola* and *Colletotrichum* spp., and to a lesser degree with *M. oryzae*, is significantly higher compared  
2 to the genome-wide average (Supplementary Fig. 6). Taken together, we hypothesize that the *CTB* cluster  
3 as a whole was transferred multiple times followed by species-specific evolutionary trajectories involving  
4 frequent gene loss. However, we cannot rule out the alternative hypothesis where *CTB*-like clusters  
5 identified in this study are the result of strong purifying selection of an ancient SM cluster, which was  
6 followed by frequent gene loss in nearly all other sequenced fungi. The outcome of reconciliation analyses  
7 depend heavily on the relative costs assigned to the various evolutionary events under consideration,  
8 namely horizontal gene transfer, gene duplication and gene loss<sup>20</sup>. Nonetheless, either scenario highlights  
9 the extraordinary evolution of the *CTB* cluster. Although *C. acutatum* has *CTB* cluster orthologs and is  
10 known to secrete a red SM *in vitro*<sup>21</sup>, we were unable to identify cercosporin in *C. acutatum* or sister  
11 species *C. fioriniae*. However, it is not unlikely that cercosporin, or a comparable SM, is only synthesized  
12 under specific experimental conditions, a feature typically observed for SM biosynthesis.

### 13 **Extension of the predicted cercosporin biosynthetic cluster based on microsynteny.**

14 Besides the microsynteny of *CTB* genes between species, we also observed a striking level of similarity  
15 outside of the predicted *CTB* cluster to *C. beticola* *CBET3\_00845* on the 3' end of the cluster (Fig. 1, 3).  
16 Considering the observed microsynteny and the significant co-expression of syntenic genes in *C.*  
17 *higginsianum*<sup>22</sup>, we hypothesized that the *C. beticola* *CTB* cluster is significantly larger than previously  
18 described<sup>10</sup>. To test this, we first determined the relative expression of all established *C. beticola* *CTB*  
19 genes as well as a number of flanking genes (*CBET3\_00828* to *CBET3\_00848*) under light (cercosporin-  
20 inducing) compared to dark (cercosporin-repressing) conditions, which showed that all candidate *CTB*  
21 genes on the 3' flank were induced in the light except *CBET3\_00846* and *CBET3\_00848* (Supplementary  
22 Table 7). Functional annotation of these novel, induced genes revealed one non-conserved phenylalanine  
23 ammonia lyase, the major facilitator superfamily transporter CFP<sup>23</sup> (*CBET3\_00841*), a candidate GA<sub>4</sub>  
24 desaturase (*CBET3\_00842*), a candidate dehydratase (*CBET3\_00843*),  $\beta$ -ig-h3 fasciclin (*CBET3\_00844*),

1 laccase (CBET3\_00845) and protein phosphatase 2A (CBET3\_00847; Supplementary Table 7; 8), which  
2 have functions associated with multi-domain enzymes or polyketide biosynthesis in fungi or bacteria<sup>10,24-</sup>  
3 <sup>29</sup>. To confirm individual gene contributions for cercosporin production, we generated single gene deletion  
4 mutants of all candidate genes from *CBET3\_00840* to *CBET3\_00846* and tested their ability to produce  
5 cercosporin. These assays showed that cercosporin production in  $\Delta$ *CBET3\_00844* and  $\Delta$ *CBET3\_00845*  
6 mutants was completely abolished, while  $\Delta$ *CBET3\_00842* mutants accumulated a red, cercosporin-like  
7 metabolite that migrated differently in potato dextrose agar (PDA) culture plates and thin layer  
8 chromatography (TLC), and exhibited a different profile obtained via high-performance liquid  
9 chromatography (HPLC) (Fig. 4). Complementation of mutants restored the ability to produce cercosporin,  
10 validating their role in cercosporin biosynthesis (Fig. 4). All other mutants produced compounds with HPLC  
11 profiles similar to cercosporin (Supplementary Fig. 7), suggesting these genes are not involved with  
12 cercosporin biosynthesis. Taken together, these results corroborate our hypothesis that the *CTB* cluster  
13 extends to *CBET3\_00845* at the 3' side and includes at least three novel *CTB* genes as well as *CFP*.  
14 Consequently, we propose naming genes *CBET3\_00842*, *CBET3\_00844* and *CBET\_00845* as *CbCTB9* to  
15 *CbCTB11*, respectively (Supplementary Table 7).

#### 16 **Pre-cercosporin isolation and characterization**

17 To characterize the red metabolite that accumulated in the  $\Delta$ *CTB9* mutant, an ethyl acetate extract of the  
18 collected mycelia was analyzed by reverse-phase HPLC. At 280 nm, a single peak was observed and  
19 compared to a reference sample of cercosporin produced by *C. nicotianae*. The retention time of this peak  
20 was shorter than that of cercosporin (Fig. 5a) suggesting a more polar metabolite. Comparison of the UV-  
21 vis spectra (Fig. 5b) of the two compounds revealed nearly identical chromophores, suggesting close  
22 structural relation. The exact mass of the metabolite from the  $\Delta$ *CTB9* mutant was determined ( $m/z =$   
23  $537.1762$ ,  $[M+H^+]$ ), consistent with the elemental composition  $C_{29}H_{29}O_{10}$ . This mass is 2 Da greater than  
24 that of cercosporin (+2 hydrogens), which led to a proposed structure for pre-cercosporin (Fig. 5c).

1 Alternative hydroquinones of cercosporin could be excluded simply on the basis of the UV-vis spectral  
2 information and chemical instability. The presence of a free phenol in pre-cercosporin in place of the  
3 unusual 7-membered methylenedioxy of cercosporin is consonant with the red shift of the long  
4 wavelength  $\lambda_{\max}$  and the shorter HPLC retention time.

5 To firmly support the tentative structure of pre-cercosporin, the crude extract obtained as above  
6 was further purified by reverse-phase HPLC and examined by  $^1\text{H-NMR}$  spectroscopy. Immediately evident  
7 in the spectrum was the absence of the methylenedioxy singlet at  $\delta 5.74$  diagnostic of cercosporin, but the  
8 appearance of a new  $-\text{OCH}_3$  at  $\delta 4.28$  and a phenol  $-\text{OH}$  at  $\delta 9.25$ . Consistent with the new asymmetry in  
9 pre-cercosporin, two strongly hydrogen-bonded *peri*-hydroxy groups could be seen far downfield at *ca.*  
10 15 ppm and two aryl hydrogens were observed at  $\delta 6.92$  and  $\delta 6.87$ . That these latter resonances are  
11 observed only in pairs as are the two side chain methyl doublets at *ca.* 0.6 ppm and the doubling of other  
12 signals imply that pre-cercosporin is formed as a single atropisomer having a helical configuration likely  
13 identical to that of cercosporin, although it is conceivable CTB9 also sets the final stereochemistry.

#### 14 ***Cercospora beticola* CBET3\_00841 (CbCFP) is required for auto-resistance to cercosporin**

15 To date, no *CTB* cluster gene has been shown to be involved with cercosporin auto-resistance<sup>10</sup>. However,  
16 targeted disruption of *C. kikuchii* *CFP* conferred enhanced sensitivity to exogenously-applied  
17 cercosporin<sup>23</sup>. To confirm if the *C. beticola* *CFP* homolog *CbCFP* also mediates resistance to cercosporin,  
18 we compared the growth of the WT versus  $\Delta\text{CbCFP}$  and ectopic mutants in cercosporin-amended liquid  
19 media assays (Supplementary Fig. 8). In the absence of cercosporin,  $\Delta\text{CbCFP}$  exhibited enhanced growth  
20 compared to the wild-type, and to a lesser extent at 1.0  $\mu\text{M}$  cercosporin. However, individual knock-out  
21 mutants of  $\Delta\text{CbCFP}$  grew to  $\sim 40\%$  of wild-type in 100  $\mu\text{M}$  cercosporin. Complemented mutants exhibited  
22 enhanced growth compared to their progenitor knock-out strains in 100  $\mu\text{M}$  cercosporin. All assays were

1 repeated with multiple  $\Delta CbCFP$  mutant strains, producing similar results. Thus, the *CTB* cluster gene *CbCFP*  
2 is required for cercosporin tolerance in *C. beticola*.

### 3 **Discussion**

4 Several hypotheses exist for the maintenance of SM biosynthetic genes as clusters. In one, unlinked SM  
5 pathway genes are at a greater risk for dissociation during meiotic recombination<sup>30</sup> or chromosomal  
6 rearrangements<sup>31</sup>. Additionally, clustering may facilitate strict coordination of gene expression, which may  
7 be particularly important during the biosynthesis of SMs that have potentially toxic intermediates<sup>32</sup>.  
8 Besides the maintenance of genome and cellular integrity, clustering may promote or be a consequence  
9 of horizontal transfer. Horizontal gene transfer of entire biosynthetic gene clusters has been reported<sup>33-</sup>  
10 <sup>36</sup>, but not to the extent and frequency observed in this study. The unprecedented number of horizontal  
11 transfers of the ancient *CTB* cluster specifically among plant pathogens suggests that it was critical for  
12 disease development in diverse pathosystems. The *CTB*-like clusters in *C. higginsianum* and *C. graminicola*  
13 were reported as one of the few SM clusters between these species that are microsyntenic<sup>22</sup>. Moreover,  
14 O'Connell detected specific upregulation of the *CTB*-like cluster in *C. higginsianum* during colonization of  
15 *Arabidopsis*<sup>22</sup>. Indeed, nine of 14 *C. higginsianum* *CTB*-like genes were among the top 100 most highly  
16 expressed genes *in planta*. Recent analysis of natural selection processes in *C. graminicola* identified  
17 orthologs of *CTB* genes *CTB1* and *CFP* as one of ~80 genes undergoing significant positive selection<sup>37</sup>,  
18 further suggesting a role in pathogenicity. We were not able to detect cercosporin production in *C.*  
19 *acutatum* or *C. fioriniae* *in vitro*, and thus future research is directed to examine *in planta* biosynthesis as  
20 well as investigate the potential role of this cluster in *Colletotrichum* pathogenicity.

21 The extensive microsynteny outside of the established *CTB* cluster prompted us to test whether  
22 the flanking genes in *C. beticola* are also required for cercosporin biosynthesis. Notably, we observed that  
23 these flanking genes, similar to the established *CTB* genes, were up-regulated under cercosporin-inducing  
24 conditions. Furthermore, targeted gene replacement of *CbCTB10* and *CbCTB11* completely abolished

1 cercosporin biosynthesis while replacement of *CbCTB9* resulted in the accumulation of a new, red  
2 metabolite. We thus conclude that the *CTB* cluster is significantly larger than previously described<sup>10</sup>. Our  
3 phylogenomics approach further revealed the presence of a duplicated, *CTB*-like cluster encompassing a  
4 similar aggregation of genes. It is noteworthy that the presence of this duplicated cluster in *C. beticola*  
5 and *C. berteroae*, and its absence in *C. zea-maydis* (JGI MycoCosm), coincides with the ability to produce  
6 beticolin, another non-host specific toxin which causes plant cell membrane disruption<sup>38,39</sup>. Future  
7 research will be directed to determine the function of this gene cluster in the *C. beticola*/sugar beet  
8 interaction.

9         The isolation and characterization of a new intermediate in the cercosporin biosynthetic pathway,  
10 pre-cercosporin, strongly suggests that formation of the unique 7-membered methylenedioxy bridge in  
11 the final product is the result of a two-step process. First, one of two precursor aryl methoxyl groups is  
12 oxidatively removed (possibly by CTB7, a flavin-dependent oxidoreductase), followed by the action of  
13 CTB9, an apparent  $\alpha$ -ketoglutarate-dependent dioxygenase annotated as similar to GA4 desaturase. In  
14 contrast, a single cytochrome P450 is known to convert two aryl *ortho*-methoxyl groups into the relatively  
15 more common 5-membered methylenedioxy group in alkaloid biosynthesis<sup>40</sup>. A tentative cercosporin  
16 biosynthesis scheme was recently proposed<sup>12</sup> without knowledge of the expanded *CTB* cluster. However,  
17 in light of the new CTB9 intermediate and the potential functions of the other newly discovered *CTB* genes,  
18 the previously reported biosynthesis pathway<sup>12</sup> will have to be revised. These investigations will be  
19 reported in due course.

20         Considering the universal toxicity of perylenequinones and the similar involvement of photo-  
21 activated perylenequinones in disease development by several plant pathogens, research on cercosporin  
22 auto-resistance mechanisms may serve as a universal model towards better understanding of cellular  
23 resistance to oxidative stress<sup>41</sup>. Moreover, the incorporation of cercosporin auto-resistance genes into  
24 crop germplasm may provide durable resistance to *Cercospora* diseases in several important crop hosts

1 and reduce the reliance on fungicides for disease management. Unlike resistance proteins that recognize  
2 pathogen protein effectors that can evade recognition with as little as a single amino acid exchange<sup>42</sup>,  
3 resistance to SMs is likely to be more durable because pathogen populations cannot easily alter SM  
4 structure to avoid recognition and cercosporin mutants of most *Cercospora* spp. are significantly less  
5 virulent<sup>23,43-45</sup>. Consequently, the search for SM auto-resistance genes within SM clusters has significant  
6 potential to lead to new developments for engineering disease resistance in crop plants against diverse  
7 fungal pathogens.

## 1 **Methods**

### 2 **Data access**

3 The *C. beticola* genome sequence, assembly and annotation as well as transcriptome reads are deposited  
4 at the National Center for Biotechnology Information (NCBI) SRA as BioProject PRJNA270309. NCBI Locus  
5 Tag Prefix *CB0940* replaces the *CBET3* Locus Tag Prefix that is used throughout this manuscript. A browser  
6 of this genome assembly is available at <http://bioinformatics.psb.ugent.be/orcae/overview/Cerbe/>.  
7 Custom Perl and R codes are available through a public GitHub repository:  
8 <https://github.com/rdejonge/genomics>, and all supplementary files are available through *figshare*; doi:  
9 10.6084/m9.figshare.4056522.

### 10 ***Cercospora* spp. genome sequencing**

11 Genomic DNA of *C. beticola* strain 09-40 was isolated using the CTAB method from mycelia scraped from  
12 the surface of V8 juice agar Petri plates<sup>46</sup>. Library preparation of three genomic libraries with increasing  
13 insert size (500 bp, 5 Kbp and 10 Kbp) and subsequent paired-end (PE) and mate-pair (MP) genome  
14 sequencing was performed by BGI Americas Corporation (BGI) via the Illumina platform. A total of  
15 ~11,100,000 high-quality filtered sequence reads with an average length of 100 bp were generated for  
16 the 500 bp PE library, ~23,500,000 reads of 50 bp length were derived from the 5 Kbp MP library, and a  
17 total of ~16,800,000 reads of 90 bp length were obtained from the 10 Kbp MP library. Total sequence  
18 output was ~3.8 Gbp, corresponding to an estimated 100-fold coverage. In the first stage of the genome  
19 assembly, SOAPdenovo (version 2.223) was used with the following command: “*SOAPdenovo-63mer all*  
20 *—s config.txt —K 51 —R —o soapfinal.fa —p 8 —L 200*” to assemble contigs and scaffolds incorporating  
21 all three libraries as input. The libraries were ranked according to insert size, using the smallest insert  
22 library as ranked first. Only the smallest insert library was used for all stages of the assembly with option  
23 “*asm\_flags=3*”, the other two libraries were only used for scaffold assembly with option “*asm\_flags=2*”.

1 For the second stage of the genome assembly, optical mapping was used to scaffold sequence reads.  
2 Optical maps were generated on the Argus System by BGI and sequences were placed using MapSolver  
3 (version 3.2.2; Opgen). BioNano Genomics optical maps (genome maps) were generated on the Irys  
4 System (version 9; BioNano Genomics) at the Nucleomics Core facility (Vlaams Instituut voor  
5 Biotechnologie), and sequences were placed using the IrysView software. Recommendations by the  
6 software packages were followed for scaffold placement. The resulting alignment maps were  
7 subsequently compared visually, and accordingly an AGP-like (A Golden Path) file was constructed  
8 manually, applying a majority-consensus ruling wherever necessary that details the placement (position  
9 and orientation) of all sequences (Supplementary File 1). Using the custom Perl script *parse\_agp-*  
10 *like\_to\_fasta.pl*, the AGP-like file was used to construct 12 supercontigs (possibly chromosomes) ranging  
11 in size from ~227 Kbp to ~6.2 Mbp. We then applied Pilon (version 1.7;  
12 <http://www.broadinstitute.org/software/pilon>) for automated assembly improvement. To this end, the  
13 sequencing reads from all three libraries (MP libraries were reverse complemented prior to mapping)  
14 were aligned to the genome assembly using Bowtie<sup>47</sup> (version 2.1.0) implementing default parameters for  
15 end-to-end mapping (i.e. “*—end-to-end, --sensitive*”, with the exception of insert length, that was  
16 adjusted accordingly for each library). Pilon was run subsequently with default parameters, incorporating  
17 the mapped PE library as “frags” and the mapped MP libraries as “jumps.”

18 Repetitive sequences were identified by RepeatMasker (version open-4.0.3;  
19 <http://www.repeatmasker.com>) using two distinct libraries: 1) the RepBase repeat library  
20 (<http://www.girinst.org/server/RepBase/index.php>, obtained on October 23, 2013) and 2) a *C. beticola*  
21 specific repeat library constructed using the RepeatModeler (version 1.0.7;  
22 <http://www.repeatmasker.org/RepeatModeler.html>). A total of 45 repeat families were found by  
23 RepeatModeler, of which 13 could be classified (Supplementary File 2).

1        *Cercospora berteroae* strain CBS 538.71 was obtained from Centraal Bureau voor Schimmelcultures  
2 (CBS) and cultivated on Petri plates containing potato dextrose agar (PDA; Difco). High-quality DNA was  
3 extracted using the CTAB method<sup>48</sup>. Library preparation (500 bp) and subsequent paired-end (PE) genome  
4 sequencing was performed by BGI via the Illumina platform. A total of 31 million high-quality filtered  
5 sequence reads with an average length of 100 bp were generated. A draft genome assembly was  
6 constructed using SOAPdenovo (version 2.04), applying default parameters and K-mer length 51.

### 7 **Transcriptome sequencing**

8 To aid in gene prediction and discover gene expression patterns under specific conditions, three RNA  
9 samples were prepared for RNA sequencing; one from *in vitro* cultured *C. beticola* tissue and two from *C.*  
10 *beticola* infected sugar beet seedlings at four and seven days post inoculation (DPI). Total RNA was  
11 extracted from flash-frozen fungal and/or plant material using a Qiagen total RNA extraction kit as  
12 described<sup>49</sup>. Library preparation and sequencing were performed at BGI, which resulted in the generation  
13 of ~12,800,000 44,500,000 and 44,800,000 PE reads of length 90 bp and fragment size of 200 bp for the  
14 *in vitro*, the 4DPI *in planta* and the 7 DPI *in planta* libraries respectively.

15        Genome-guided Trinity assembly<sup>50</sup> of sequencing reads was subsequently performed as described  
16 by the software manual, with minimal intron length of 20 bp and the GSNAP aligner. Transcript assemblies  
17 were subsequently generated using PASA2 (version r20140417). We applied another transcript assembly  
18 approach simultaneously by mapping the RNA-Seq reads to the reference genome using Tophat (version  
19 2.0.8b; <http://ccb.jhu.edu/software/tophat/index.shtml>), converting these to raw transcript assemblies  
20 using Cufflinks (version 2.1.1; <http://cufflinks.cbc.umd.edu/>) and final selection of the best assemblies  
21 using TransDecoder (version 2014-07-04; <http://transdecoder.sourceforge.net/>).

### 22 **Gene prediction and curation**

1 EvidenceModeler<sup>51</sup> was used to predict protein-coding genes by integrating protein-coding evidence from  
2 multiple sources according to specified weights. First, Augustus<sup>52</sup> parameters (version 2.5.5) were trained  
3 locally with the *autoAug.pl* script included in the Augustus software package, using the assembled Trinity  
4 transcripts sequences as training input as well as hints in the final gene prediction step. Secondly,  
5 GeneMarkES<sup>53</sup> (version 2.3c) was used in self training modus, optimizing coding parameters for the full  
6 genome sequence. Protein similarity was detected by the alignment of proteins from the related fungal  
7 species *Z. tritici*, *P. nodorum*, *L. maculans*, *C. heterostrophus* and *C. zea-maydis* against the genome  
8 sequence using the Analysis and Annotation Tool (AAT) package<sup>54</sup> (version r03052011). Protein sequences  
9 from these related fungi were derived from the MycoCosm platform at the Joint Genome Institute (JGI;  
10 <http://genome.jgi-psf.org/programs/fungi/index.jsf>). For integration of the various protein-coding  
11 sources with the EvidenceModeler, we ranked all sources according to their expected accuracy and  
12 importance. From high to low the ranking was: genome-aligned Trinity transcripts (with PASA), Cufflinks-  
13 derived TransDecoder transcripts, trained Augustus *ab initio* predictions, aligned protein sequences and  
14 self-trained GeneMarkES *ab initio* predictions. These initial EvidenceModeler transcripts were  
15 substantially further improved by manual curation on the WebApollo<sup>55</sup> (version 2013-05-16) platform.  
16 Approximately 10,500 gene models were visually analyzed and updated according to the available  
17 evidence as needed. The updates included adjustments to splice sites (e.g. alternative donor or acceptor  
18 sites), exon usage, five prime and three prime UTRs (untranslated regions) and the addition of alternative  
19 transcripts where supported by RNA-Seq evidence. A majority-consensus ruling was used for the updates,  
20 considering all evidence tracks. For transfer of the final gene predictions to newer versions of the  
21 assembly, the map2assembly algorithm from Maker was used routinely (version 2.28;  
22 <http://www.yandell-lab.org/software/maker.html>).

23 To investigate the predicted gene models in more detail, we calculated a number of statistics  
24 based on the gene structures. For this purpose, all gene models were stored in GFF3 format according to

1 the specifications at The Sequence Ontology Project (<http://www.sequenceontology.org/gff3.shtml>). The  
2 *gff3\_gene\_prediction\_file\_validator.pl* Perl script, part of the EvidenceModeler package was routinely  
3 used for verification of GFF3 file format. GFF3 gene statistics, e.g. the mean/median gene length, the  
4 number of exons, CDSs or UTRs, were calculated using the custom Perl script, *GFF3stats.pl*.

#### 5 **Protein function characterization**

6 For functional characterization of the predicted protein sequences, hardware-accelerated BLASTp on a  
7 DeCypher machine (TimeLogic; Carlsbad, USA) was used to identify homologous proteins in the non-  
8 redundant (nr) protein database obtained at the NCBI. InterProScan (version 5.44;  
9 <http://www.ebi.ac.uk/Tools/pfa/iprscan5/>) was used to identify conserved protein domains. The results  
10 of both analyses were imported into Blast2GO<sup>56</sup> and used to generate single, uniquely functional  
11 annotations for each protein as well as a list of all associated gene ontology (GO) terms.

#### 12 **Secondary metabolite cluster identification, characterization and visualization**

13 SM clusters were identified in the genome sequence of *C. beticola* and that of related fungi using  
14 antiSMASH2<sup>15</sup> (version 2.1.0; <https://bitbucket.org/antismash/antismash2/>). To generate antiSMASH2-  
15 required EMBL formatted genome files, the GFF3 gene features files in combination with the respective  
16 genome sequences were converted to the EMBL sequence format using the custom Perl script  
17 *GFF3\_2\_EMBL.pl*. Subsequently, antiSMASH2 was run with default parameters, allowing for the  
18 identification of PKS SM clusters, NRPS SM clusters, Hybrid PKS-NRPS SM clusters, terpene cyclase (TC)  
19 SM clusters, siderophores SM clusters and lantipeptide SM clusters. SM clusters that showed similarity to  
20 a mixture of these clusters or only a minimal set of homologous protein domains were depicted as “other.”  
21 In addition, DMAT, for dimethylallyl tryptophan synthase clusters were identified by screening the  
22 InterProScan results for Pfam domain PF11991.

#### 23 **Secondary metabolite phylogenetic analyses**

1 For phylogenetic analyses of the type I polyketide enzymes we used Mafft (version 7.187), applying global  
2 alignment (--globalpair) and a 1000 cycles of iterative refinement (--maxiterate 1000), to align full-length  
3 sequences as well as selected domains of all PKS enzymes that were identified by antismash2 in the  
4 genome sequences of the six Dothideomycetes: *C. beticola*, *D. septosporum*, *Z. tritici*, *L. maculans*, *P.*  
5 *tritici-repentis* and *P. nodorum*, and one Eurotiomycete: *Aspergillus nidulans*. In addition, previously  
6 characterized polyketide synthases (Supplementary Table 3) were included for reference. Prior to  
7 phylogenetic tree reconstruction, the alignments were trimmed with TrimAl<sup>57</sup> (version 1.2). Maximum  
8 likelihood phylogenetic trees were determined with RaxML (version 8.1.3), applying rapid bootstrapping  
9 (-# 100) and automated protein model selection (-m PROTGAMMAAUTO). Final trees were prepared  
10 online using EvolView<sup>58</sup>. Species tree topologies were built with Cytoscape<sup>59</sup> webserver by uploading the  
11 predicted proteomes of 48 published Ascomycete fungi (Supplementary Table 4).

12 For phylogenetic tree reconciliation analyses of the protein and species trees, the protein trees  
13 were pre-processed with treefixDTL<sup>60</sup> (version 1.1.10) to minimize errors introduced during tree  
14 reconstruction. TreefixDTL is able to correct phylogenetic trees in the presence of horizontal gene  
15 transfer. Reconciliation analyses as well as rooting were conducted in NOTUNG<sup>19</sup> according to the  
16 instructions (version 2.8 beta).

### 17 **Secondary metabolite cluster alignment visualization**

18 For comparative analyses of the secondary metabolite clusters, we used the R-package *genoPlotR*<sup>61</sup>  
19 (version 0.8.2; <http://genopltr.r-forge.r-project.org/>). To this end, individual clusters were extracted  
20 from the genome sequence using the Perl script *get\_seq\_by\_id.pl*, using the start or stop positions of the  
21 flanking genes in the cluster as extremities. The resulting sequences were then aligned using the bl2seq  
22 (BLAST two sequences) algorithm, part of the BLAST toolkit (version 2.2.26). Also, transcripts locations  
23 (CDS start and stop coordinates specifically) were extracted from the GFF3 files using  
24 *gff3CDS\_2\_genopltr.pl*, and adjusted according to the start and end coordinates of the extracted cluster

1 sequence, by means of the Unix command line tool Awk. We then used custom R code (example in  
2 *genoPlotR.R*) to parse these input files and generate cluster alignment figures.

### 3 **Genome-wide gene cluster microsynteny and protein identity analysis**

4 Genome-wide gene-by-gene cluster analyses were performed using the custom Perl script  
5 *calClusterSimilarity.pl*, and plotted using ggplot2 in R using *synteny.R*. As input, this pipeline takes the  
6 typical output of an orthoMCL analysis, reformatted by *analyseOrthoMCL.pl*. In short, it requires each  
7 proteinId to have an associated clusterId. Furthermore, it requires properly formatted GFF3 files for each  
8 genome that are used to associate location of protein-coding genes and their flanks. Last but not least,  
9 the number of flanking genes to be used can be chosen freely, but must be set ODD. For the analyses  
10 presented in Supplementary Figure 5, a cluster size of 30 was set. Genome-wide protein-by-protein best-  
11 BLAST percent identities were derived from the similarities table prepared during orthoMCL analyses and  
12 subsequently plotted in R using *pairwise\_pident\_boxplots.R*.

### 13 **Gene expression analysis**

14 To investigate the expression of cercosporin cluster genes, *C. beticola* was grown in a 250 ml Erlenmeyer  
15 flask containing 100 ml potato dextrose broth (PDB; Difco) in the light and dark, which are conditions that  
16 promote and repress cercosporin production, respectively. Total RNA was isolated using TRIzol  
17 (ThermoFisher) following the manufacturer's instructions followed by an on-column Dnase treatment  
18 (Qiagen). Total RNA was used for cDNA synthesis using an oligo-(dT) primer and the SuperScript II reverse  
19 transcriptase kit (Invitrogen) following manufacturer's instructions. The resulting cDNA was used as a  
20 template for quantitative polymerase chain reaction (qPCR). Selected genes were queried for expression  
21 using the Power SYBR Green PCR Master Mix (Applied Biosystems) using a PTC-2000 thermal cycler (MJ  
22 Research) outfitted with a Chromo4 Real-Time PCR Detector (Bio-Rad) and MJ Opticon Monitor analysis  
23 software version 3.1 (Bio-Rad). Primers for gene expression are listed in Supplementary Table 9.

## 1 **Transformation and disruption of target genes**

2 Split-marker PCR constructs for targeted gene replacement were prepared as described<sup>46</sup> using genomic  
3 DNA of 10-73-4 and 09-40 wild-type *C. beticola* and pDAN as PCR templates. Selected mutants were  
4 complemented using pFBT005, which encodes resistance to nourseothricin and allowed us to clone our  
5 gene of interest between the ToxA promoter and TrpC terminator using PaeI and NotI (Promega)  
6 restriction sites. Primers for split-marker and complementation constructs are listed in Supplementary  
7 Table 9.

8 A 5 mm plug was taken from the actively growing zone of *C. beticola* on PDA. Liquid cultures were  
9 initiated by grinding the plug with 500  $\mu$ L Fries media<sup>46</sup>, which was subsequently transferred to a 125 mL  
10 flask containing 50 ml Fries media. Flasks were wrapped in aluminum foil and shaken at 150 rpm at 21 °C  
11 for four days. Cultures were then transferred to a sterile blender cup, ground for 10 s, and transferred to  
12 a 500 mL flask containing 200 mL Fries media. Cultures were grown as described above for an additional  
13 24 h after which mycelium was harvested with two layers of Miracloth (Calbiochem) using a Büchner  
14 funnel. The mycelium was rinsed with mycelium wash solution (0.7M KCl, 10mM CaCl<sub>2</sub> · 2H<sub>2</sub>O), broken  
15 into small pieces with a sterile spatula, transferred to a deep Petri dish containing 40 mL of osmoticum  
16 (20 g L<sup>-1</sup> lysing enzymes from *T. harzianum* (Sigma-Aldrich) and 12.5 g L<sup>-1</sup> Driselase (Sigma-Aldrich), and  
17 incubated at 30 °C for 6 to 14 h while shaking at 50 rpm. Protoplasts were harvested by filtering the  
18 osmoticum solution through two layers of Miracloth and collected by centrifugation (2000 x *g* for 5 min).  
19 After a washing step with 15 mL STC (1 M Sorbitol, 10 mM Tris-HCl pH 7.5, 10 mM CaCl<sub>2</sub>), protoplasts were  
20 adjusted to 10<sup>8</sup> mL<sup>-1</sup> with STC:PEG (4:1) and divided into 200- $\mu$ L aliquots. Subsequently, PEG-based  
21 transformation was performed essentially as described<sup>46</sup> using 70  $\mu$ g of each construct or 70  $\mu$ g of  
22 complementation plasmid linearized with Apal (Promega) per 200  $\mu$ L protoplast aliquot.

## 23 **Cercosporin production**

1 To screen for the ability to produce cercosporin, 10 mm plugs were transferred from the growing edge of  
2 *C. beticola* 10-73-4 wild-type, mutant and complemented mutant colonies to 100 mm x 15 mm Petri plates  
3 (Falcon; Oxnard, USA) that contained 10 ml PDA. After an incubation period of 6 days at 21 °C with a  
4 natural light-dark cycle, five 10 mm (dia) plugs of PDA containing *C. beticola* mycelium were collected and  
5 extracted with ethyl acetate for 18 h in the dark at 4 °C. The extracts were filtered and dried under a  
6 stream of nitrogen (30 °C). The dried extracts were re-dissolved in 25% acetonitrile in 1% aqueous acetic  
7 acid and fractionated by reverse-phase HPLC on a Waters 600 HPLC system fitted with a Waters radial pak  
8 column (8 mm x 120 mm, 8 µm). Injections were 200 µl. Solvent A was 1% (v/v) aqueous acetic acid and  
9 solvent B was acetonitrile. Starting conditions were 25% A 75% B, hold for 1 min, a linear gradient to 100%  
10 B in 19 min, hold for 5 min, and return to initial conditions in 5 min (Fig. 4b). Under these conditions,  
11 cercosporin had a retention time of ca. 15.4 min. Where needed, extracts were also subjected to thin-  
12 layer chromatography (TLC) on Silica HF Plates pre-coated with calcium phosphate and reactivated as  
13 described Balis and Payne<sup>62</sup>. Plates were developed in hexane: 2-propanol (8:2, v/v) and visualized by UV  
14 fluorescence. For cercosporin isolation, multiple mycelial mats were collected and extracted two times  
15 with ethyl acetate (4 °C, dark). After filtration, the extracts were partitioned against an equal volume of  
16 reagent-grade water, residual water was removed by passage through anhydrous sodium sulfate, and the  
17 extracts were dried under a stream of nitrogen (30 °C). Cercosporin was purified by TLC as described  
18 above and quantified by spectrophotometry using published extinction coefficients<sup>63</sup>.

### 19 **Pre-cercosporin isolation and characterization**

20 Mycelial plugs of *C. beticola*  $\Delta$ CTB9 were placed on top of eight “thin” PDA (Difco) plates (3.0 mL PDA per  
21 50 mm Petri plate). Cultures were incubated at 22 °C for one week under continuous light. Three separate  
22 methods were attempted to prepare crude secondary metabolite extractions. 1) PDA and mycelia were  
23 extracted with ethyl acetate for 4 min. The resulting supernatant was collected and frozen for further  
24 analysis. 2) PDA and mycelia were placed into a GenElute Maxiprep binding column (Sigma Aldrich) and

1 centrifuged at 3500 x *g* for 10 min. The flow through was collected and frozen for further analysis. 3) PDA  
2 and mycelia were placed into a GenElute Maxiprep binding column along with 15 mL ethyl acetate. After  
3 a 30 s incubation, the column was centrifuged at 2400 x *g* for 10 min. The flow through was collected and  
4 frozen for further analysis. To obtain sufficient pre-cercosporin for isolation and NMR analysis, primary  
5 extracts from all three methods were combined.

6 The combined extracts were re-suspended with water and acidified with conc. HCl. Pre-  
7 cercosporin was extracted quickly from this aqueous solution by partitioning thrice with ethyl acetate in  
8 dark, wrapping the glassware with aluminum foil. The combined ethyl acetate fractions were washed  
9 with brine, dried over anhydrous sodium sulfate and evaporated under vacuum at 30 °C. The reddish-  
10 brown residue was resuspended in methanol and filtered through 0.2 µm PTFE filters. The methanol  
11 extracts were initially analyzed by reverse phase HPLC (Fig. 5a) on an Agilent model 1200 fitted with a  
12 Kinetex XB-C18 column (4.6 mm x 75 mm, 2.6 µm, Phenomenex). Injections of 1 µl were run at 1.25  
13 mL/min on a linear gradient of 5% solvent C/95% solvent D to 95% solvent C/5% solvent D over 10.8 min,  
14 where solvent C is water + 0.1% formic acid and solvent D is acetonitrile + 0.1% formic acid.  
15 Chromatograms were monitored at 436, 280, and 210 nm, and UV-vis spectra were recorded over a range  
16 of 210-800 nm (Fig. 5b). High-resolution mass data were obtained from a Waters Acquity/Xevo-G2 UPLC-  
17 ESI-MS in positive ion mode.

18 To isolate sufficient pre-cercosporin for <sup>1</sup>H-NMR analysis, the filtered methanol extract prepared  
19 above was purified by reverse-phase HPLC on an Agilent model 1100 fitted with a Kinetex XB-C18 semi-  
20 prep column (10 mm x 250 mm, 5 µm, Phenomenex). The crude extract (10 mg/mL in methanol) was  
21 injected (generally 500 µl) and run at 4 mL/min using the following method: 20% solvent C/80% solvent D  
22 for 3 min, 20-70% solvent C over 17 min, 70-95% solvent D over 5 min, where solvent C and D were as  
23 above. Chromatograms were recorded at 436, 280, and 210 nm. The metabolite of interest was collected

1 from multiple injections, combined, and lyophilized to dryness. The purified pre-cercosporin was analyzed  
2 by UPLC-ESI-MS as described above and  $^1\text{H-NMR}$ .

3 **4,6,9-trihydroxy-1,12-bis(2-hydroxypropyl)-2,7,11-trimethoxyperylene-3,10-dione (pre-cercosporin)**

4 Data obtained at 5 °C on a Bruker AVANCE spectrometer.  $^1\text{H NMR}$  (400 MHz,  $\text{CDCl}_3$ ,  $\delta$ ): 15.24 (s, 1H), 14.93  
5 (s, 1H), 9.25 (s, 1H), 6.92 (s, 1H), 6.87 (s, 1H), 4.28 (s, 3H), 4.19, (s, 3H), 4.18 (s, 3H), 3.57 – 3.51 (m, 2H),  
6 3.42 – 3.36 (sym 5-line overlapping signal, 2H), 2.86 – 2.74 (m, 2H), 0.64 (d,  $J = 6.1$  Hz, 3H), 0.60 (d,  $J = 6.1$   
7 Hz, 3H). UPLC-ESI-HRMS: calculated for  $\text{C}_{29}\text{H}_{29}\text{O}_{10}$  [ $\text{M}+\text{H}^+$ ]: 537.1761, found [ $\text{M}+\text{H}^+$ ]: 537.1762.  $^1\text{H-NMR}$   
8 spectra (400 MHz in  $\text{CDCl}_3$ ) of pre-cercosporin and cercosporin for comparison are available as  
9 Supplementary Figures 9 and 10.

10 **Cercosporin auto-resistance**

11 To identify mutants that were sensitive to cercosporin, approximately  $10^3$  spores collected from selected  
12 strains were transferred to individual wells of a Nunc 96 well flat-bottom plate (Fisher Scientific) using  
13 Fries media as the growth medium and sealed with SealPlate (Excel Scientific). Within four hours, each  
14 well was amended with cercosporin extracted from *C. beticola* strain 10-73-4 as described above to a final  
15 concentration of 0.0, 1.0, 10.0, or 100.0  $\mu\text{M}$ . Cultures were incubated at 21°C under 24 hour light  
16 conditions. Growth was quantified daily by collecting absorbance values from each well generated from  
17 a GENios plate reader (Tecan) using XLUOR4 software (V 4.51) using a measurement wavelength of 595  
18 nm and 2x2 reads per well after vortexing the plate briefly. The absorbance value and associated standard  
19 deviation and error for each strain was determined by taking the average of 16 wells (replications) per  
20 timepoint. Each experimental run consisted of five timepoints, which were taken every 24 h starting with  
21 day 0, which were the measurements taken immediately after cercosporin treatments were added to  
22 each well, up to and including day 4. Fungal growth was calculated by subtracting the absorbance value

1 of day 0 from each timepoint. Each run was repeated at least three times with each of three individual  
2 knock-out mutant strains.

### 3 ***Colletotrichum* spp. cercosporin assay**

4 To determine whether *Colletotrichum* species were able to produce cercosporin, seven monoconidial  
5 isolates of *C. acutatum* (isolates CA1, HC50, HC72, HC 75, HC81, HC89, and HC91) and one *C. fiorinae*  
6 (3HN12) were grown on 9 cm Petri plates containing 15 ml of PDA as described above to replicate  
7 conditions that were conducive for cercosporin production *in vitro*. Seven day old cultures of each isolate  
8 were grown in a temperature controlled incubator at 25 °C with natural light. A pinkish to dark red color  
9 was visible in the media for all isolates except HC75 which had a yellow-colored pigment. Using a #2 cork  
10 borer, three plugs were removed from each isolate from the edge, middle and center of each colony and  
11 placed in small screw cap glass vials. Three plugs were also removed from an uncolonized PDA plate and  
12 included as a negative control. Cercosporin (Sigma-Aldrich) was dissolved in acetone to 100 mM and used  
13 as a positive control. There were 10 samples, seven total for each of the *Colletotrichum spp.* isolates, one  
14 containing cercosporin toxin as a positive control, and one blank plug that served as a negative control.  
15 5N KOH was added to each vial to cover the surface of the plugs and incubated on a shaking incubator at  
16 room temperature for 4 hours. Supernatants were examined for cercosporin spectrophotometrically.

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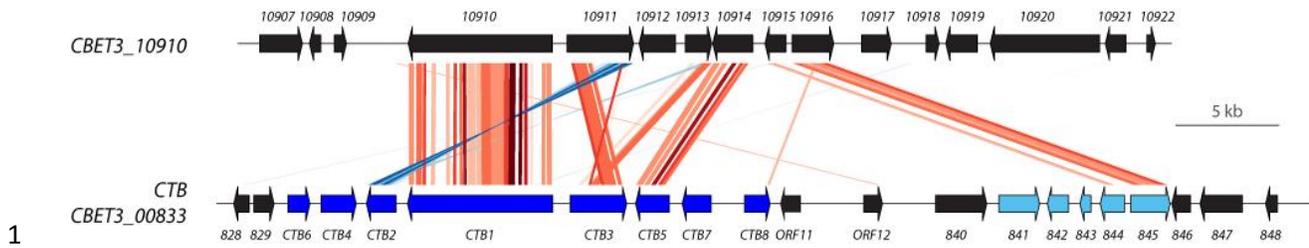
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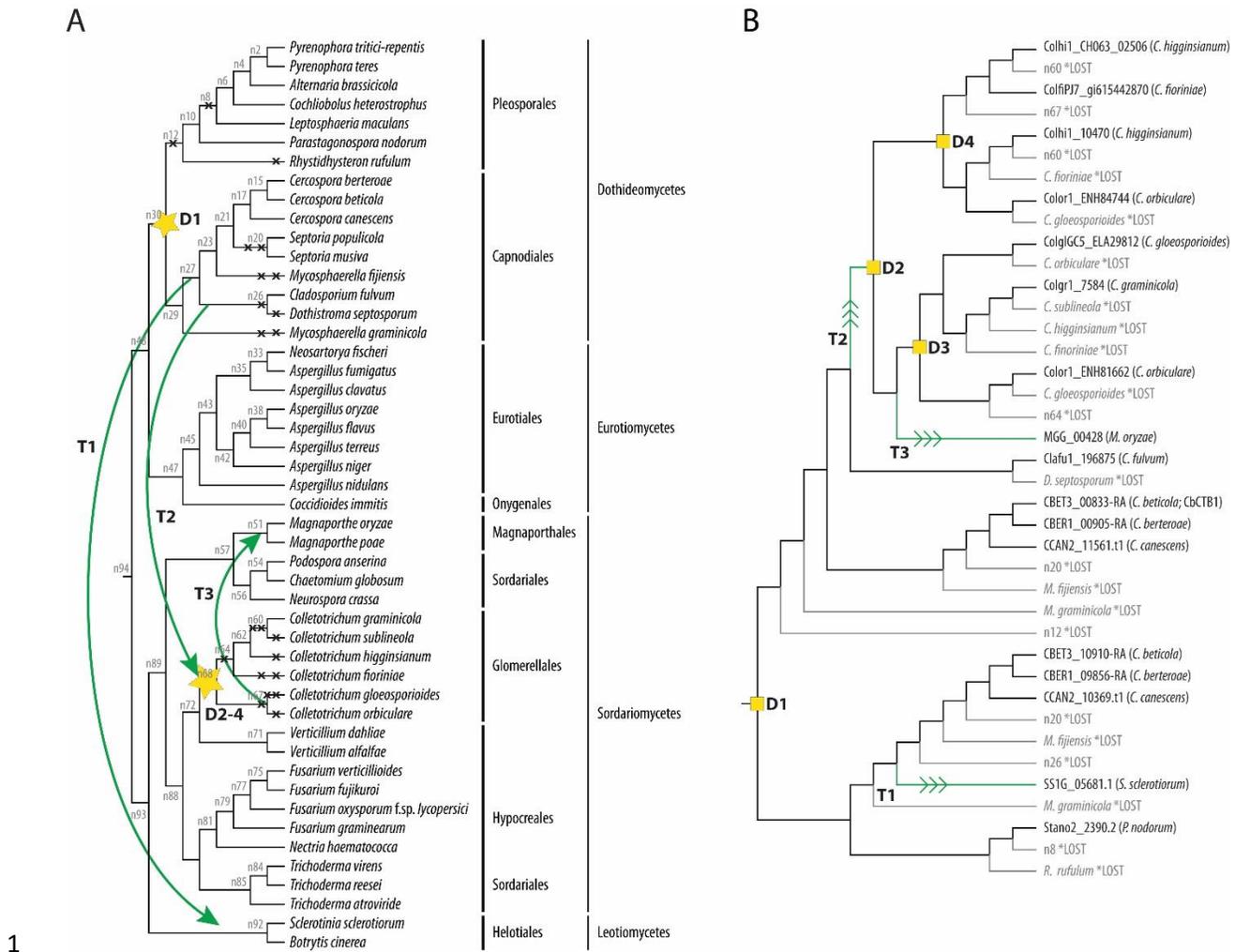
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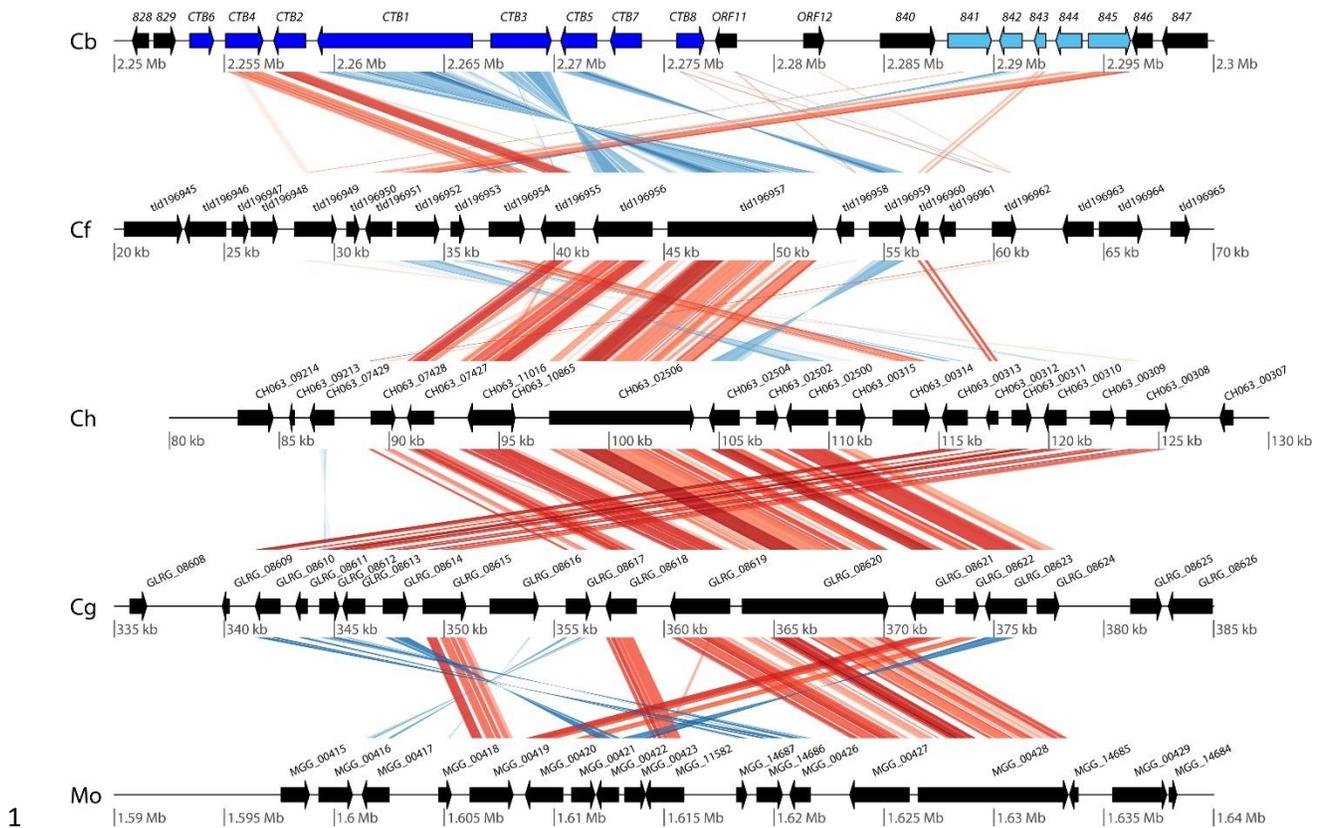
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- 5



1  
2 **Figure 1: The cercosporin biosynthetic cluster is duplicated and maintained in *C. beticola*. *CBET3\_10910***  
3 **and flanking genes are syntenic with the *CTB* cluster (*CBET3\_00833* and flanking genes) in *C. beticola*.**  
4 Alignment lines correspond to DNA fragments exhibiting significant similarity when the genomic regions  
5 comprising the gene clusters are compared with tBLASTx. Direct hits are displayed in red, whereas  
6 complementary hits are in blue. The hue of the alignments represents the percentage similarity ranging  
7 from 23 to 100 percent.

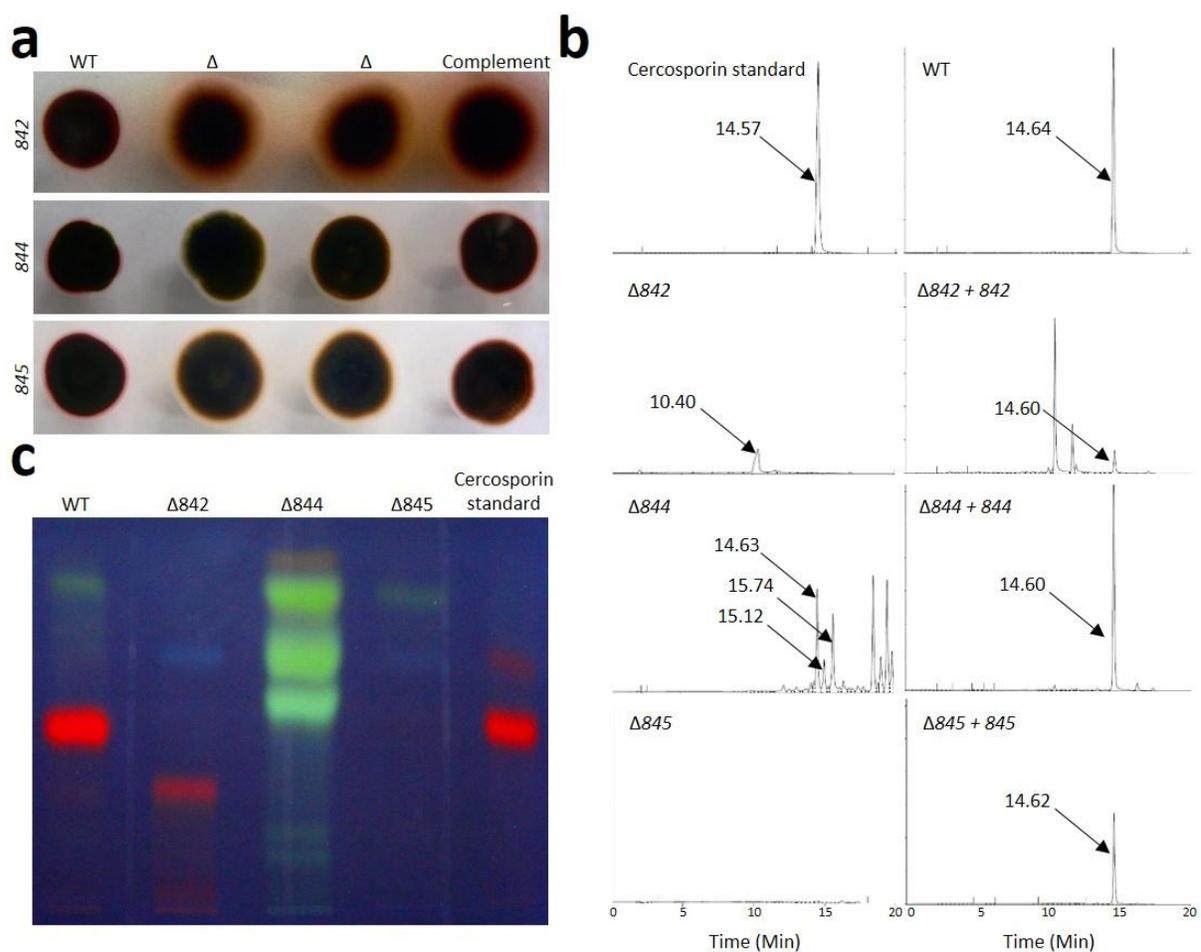


1  
2 **Figure 2: Phylogeny of *Cercospora* spp. and related Ascomycete fungi and reconciliation of CTB1**  
3 **orthologs. (a)** Cladogram showing the phylogenetic relationship of *Cercospora* spp. and 45 other  
4 sequenced fungi. The unscaled tree was constructed using CVTree<sup>59</sup>. **(b)** Tree reconciliation of CTB1. The  
5 phylogenetic tree of CTB1 determined by RaxML, corrected using the treefixDTL algorithm, and rooted  
6 and reconciled by Notung with the species tree in A to infer the number of duplications, losses and  
7 transfers. Duplication nodes are marked with yellow squares, losses are in grey and transfers are  
8 highlighted by green edges and arrow markings. Descendants of each duplication are colored according  
9 to the cross marks at the duplication nodes.



2 **Figure 3: Synteny and rearrangements of the conserved *C. beticola* cercosporin biosynthetic cluster.** The  
3 cercosporin biosynthetic cluster in *C. beticola* (Cb), marked in blue, and flanking genes are conserved in  
4 *fulvum* (Cf), *C. higginsianum* (Ch), *C. graminicola* (Cg) and *M. oryzae* (Mo). For all species the displayed  
5 identifiers are transcript IDs and the corresponding sequences can be retrieved from JGI MycoCosm or  
6 ORCAE. Alignment lines correspond to DNA fragments exhibiting significant similarity when the genomic  
7 regions comprising the gene clusters are compared with tBLASTx. Direct hits are displayed in red, whereas  
8 complementary hits are in blue. The hue of the alignments represents the percentage similarity ranging  
9 from 21 to 100 percent. Novel *C. beticola* CTB genes, identified in this study, are marked in light blue.

1



2

3 **Figure 4: Analysis of cercosporin production in *CTB* mutants of *C. beticola*.** Site-directed knock-out

4 mutants in genes *CBET3\_00842* (*CbCTB9*), *CBET3\_00844* (*CbCTB10*), and *CBET3\_00845* (*CbCTB11*) and

5 their associated complements were assayed for cercosporin production (a) visually by growth on Petri

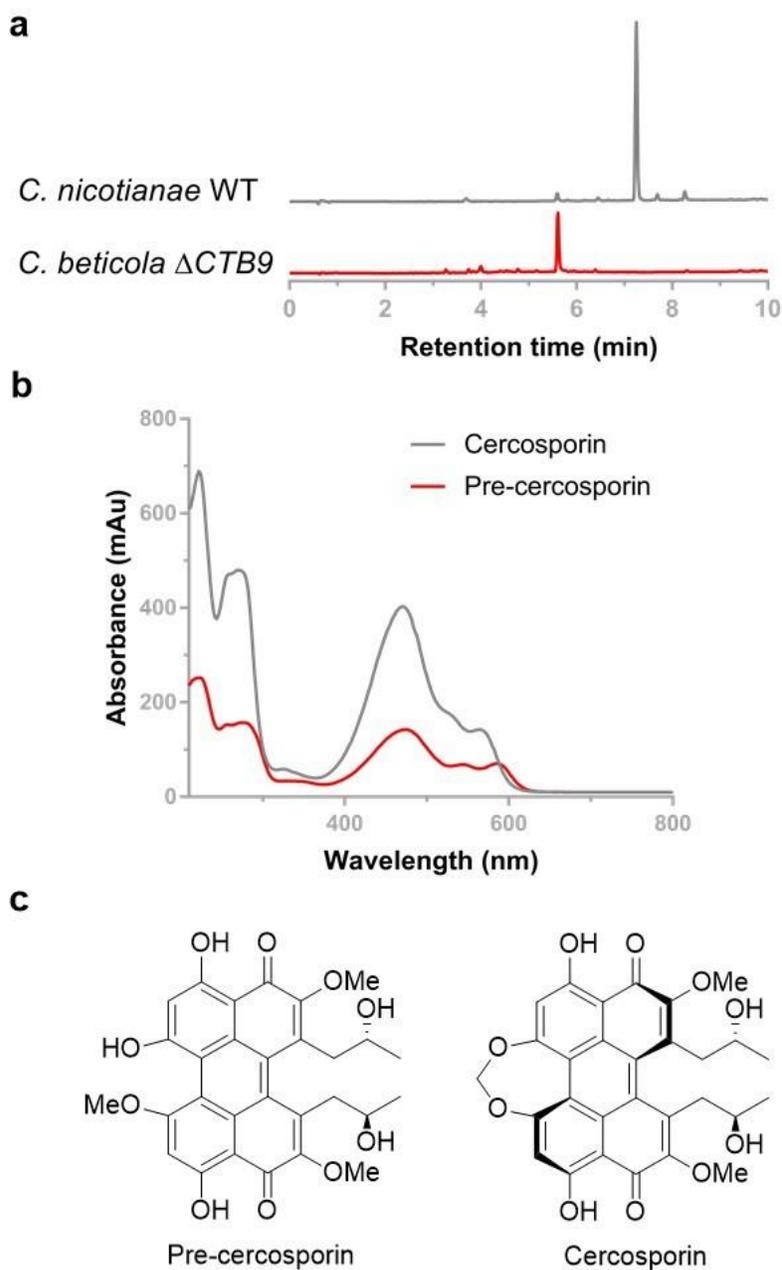
6 plates containing thin PDA where the red pigment around cultures is indicative of cercosporin, (b)

7 comparing HPLC retention times recorded at 280 nm from extracts of *C. beticola* and *C. beticola* mutants,

8 and (c) TLC. A commercial cercosporin standard and cercosporin extracted from *C. beticola* strain 10-73-

9 4 (WT) were used as controls in HPLC and TLC assays. Complemented mutants in HPLC assays are indicated

10 as Δ842+842, Δ844+844, and Δ845+845.



1

2 **Figure 5: Characterization of the metabolite isolated from *C. beticola*  $\Delta$ CTB9.** (a) HPLC retention time

3 comparison of cercosporin (gray) extracted from *C. nicotianae* and pre-cercosporin (red) extracted from

4  $\Delta$ CTB9, recorded at 280 nm. (b) Overlay of the UV-vis spectra (210-800 nm) of cercosporin (gray) and pre-

5 cercosporin (red). (c) Structures of pre-cercosporin and cercosporin (for  $^1$ H-NMR spectra, see Methods).