EXPRESSION ANALYSIS OF THE EXPANDED CERCOSPORIN GENE CLUSTER IN *CERCOSPORA BETICOLA*

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Karina Anne Stott

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Expression Analysis of the Expanded Cercosporin Gene Cluster in Cercospora beticola

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Karina Anne Stott

The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

SUPERVISORY COMMITTEE:

Dr. Gary Secor
Chair

Dr. Melvin Bolton

Dr. Zhaohui Liu

Dr. Stuart Haring

Approved:

5-18-18
Date

Dr. Jack Rasmussen
Department Chair

ABSTRACT

Cercospora leaf spot is an economically devastating disease of sugar beet caused by the fungus *Cercospora beticola*. It has been demonstrated recently that the *C. beticola CTB* cluster is larger than previously recognized and includes novel genes involved in cercosporin biosynthesis and a partial duplication of the *CTB* cluster. Several genes in the *C. nicotianae CTB* cluster are known to be regulated by 'feedback' transcriptional inhibition. Expression analysis was conducted in wild type (WT) and *CTB* mutant backgrounds to determine if feedback inhibition occurs in *C. beticola*. My research showed that the transcription factor *CTB8* which regulates the *CTB* cluster expression in *C. nicotianae* also regulates gene expression in the *C. beticola CTB* cluster. Expression analysis has shown that feedback inhibition occurs within some of the expanded *CTB* cluster genes. The partial duplication of the *CTB* cluster was not found to be light activated or subject to feedback inhibition.

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CHAPTER 1. LITERATURE REVIEW

Sugar Beet

History and Commercial Development

Sugar beet (*Beta vulgaris* spp. *vulgaris.*), a member of the family Amaranthaceae, is grown for its high sucrose content. Sugar content in sugar beet ranges from 14 to 20% (Harveson et al, 2009). Sugar beet is an economically important crop throughout the world, producing roughly 40 percent of the world's sugar (Mirvat et al, 2005). Sugar beet has a conical, typically white, fleshy tap root that on average ranges in weight from 0.5 to 1 kg (Hill and Langer, 1991). Leaves of sugar beet are large, glaborous, dark green in color and grow in a rosette (Duke, 1983). Sugar beet is a biennial that requires vernalization to produce viable seed (Letschert et al, 1994).

Sugar beet has been consumed for centuries. For example, sugar beet was mentioned by the Greek historian Herodotus as one of the vegetables consumed by the builders of the pyramids (Griffin, 1920). By 2000 B.C., the first species of *B. vulgaris* to be domesticated was chard (Harveson et al, 2009). Recordings of the first *B. vulgaris* ancestors were first mentioned around 420 BC by the Greek poet Aristophanes (Winner, 1993). These ancestors were likely wild sea beet (*B. vulgaris* ssp. *martima*), whose center of origin is debated. There are competing theories on whether the center of origin is in Europe, the Canary Islands, or Southwest Asia (Biancardi et al, 2012).

In 1600, the French agronomist Olivier de Serres reported on beetroot arriving from Italy. He said "It has a deep red root and rather thick leaves and is good to eat when prepared in the kitchen. The root is counted among choice foods." (Cooke and Scott, 1993). Later in the 17th century large rooted beets were used as fodder for cattle and other livestock (Cooke and Scott, 1993). In 1747, the German chemist Andreas Sigismund Marggraf found that beet roots produced

the same sucrose as cane sugar (Marggraf, 1749). Marggraf's research led his student Franz Karl Archard to develop a robust method of extracting sugar from beet roots, for which Archard is now recognized as the "father of the sugar beet industry" (Ministry of Agriculture and Fisheries 1931). Archard established the first sugar beet factory in 1801 at Cunern in what is now Poland.

In the early 1800s, the majority of Europe's sugar was imported from sugar cane in the West Indies. The importation of goods from the West Indies was cut off during the Napoleonic Wars which allowed for the European sugar beet industry to take hold. In France between 1810 and 1815, more than 79,000 of acres of sugar beet were in production and 300 small processing plants were built. After the Napoleonic Wars ended, more beet sugar factories were established across Europe (Harveson and Rush, 1994).

The first attempt to establish sugar beets and factories to refine sugar in the United States was in 1838 in Philadelphia, but was ultimately shut down two short years later (Harveson, 2014). The first successful sugar beet plant in the United States was founded in 1870 in Alvarado, California (Francis, 2006). By the 1950s sugar beet had been successfully cultivated in 22 states, with sugar beet grown on approximately 360,000 hectares (Draycott, 2006; Biancardi et al, 2010). Today sugar beet is grown in Oregon, Idaho, Wyoming, Colorado, Montana, North Dakota, Minnesota, Michigan, Nebraska, and California (USDA, 2015). The Red River Valley region (consisting of the region along the border between North Dakota and Minnesota) is the largest in the United States with it contributing 54 percent of total national production (McConnell, 2013).

Diseases

One of the limiting factors in sugar beet production is disease. These diseases can be caused by fungi, viruses, nematodes, oomycetes and bacteria. These pathogens vary in what part of the plant they attack, the growth stage, and the time of season.

Severe fungal foliar diseases can cause up to a 50 percent reduction in root yield and sugar content (Wolf and Verreet, 2002). The destruction of leaves by foliar pathogens induces the plant to continually regrow leaves at the expense of sugar production (Holtschulte, 2000). Some of the fungal foliar diseases include Cercospora leaf spot (*Cercospora beticola*), Ramularia leaf spot (*Ramularia beticola*), Alternaria leaf spot (*Alternaria alternate*), beet rust (*Uromyces betae*), Powdery mildew (*Erysiphe betae*) and Downy mildew (*Peronospora farinos*).

Ramularia beticola is the causal agent of Ramularia leaf spot, a disease that thrives in cool climates with high humidity (Ahrens, 1987; Asher and Handson, 2006). It is most important in Northern Europe particularly in Nordic countries such as Denmark, Sweden and Finland (Ahrens, 1987; Persson and Olsson, 2006). Symptoms of Ramularia leaf spot are circular necrotic lesions approximately 2 to 10 mm in diameter (Ahrens, 1987; Hestbjerg et al, 1994). When mature, the disease causes sunken lesions with whitish conidia sporulating at the center (Byford, 1975; Nielsen, 1991). Ramularia typically does not cause enough damage to warrant management measures in the United States. The disease is most prevalent on sugar beet seed crops in the United States (Bennet and Leach, 1971).

Alternaria alternata and *A. brassicae* are the causal agents of Alternaria leaf spot. The fungi that cause Alternaria leaf spot are found in all sugar beet growing regions (Franc, 2009). Symptoms include spotting on the leaves, which start small and expand into circular or irregular dark brown lesions ranging in size from 2 to 10 mm in diameter and can lead to defoliation (McFarlane et al, 1954). Alternaria leaf spot typically starts late in the season and is managed by proper irrigation and practices that reduce plant stress (Franc et al, 2001).

Beet rust is caused by the fungus *Uromyces betae*. Beet rust occurs in parts of the United States and Canada, most European countries, Asia, Egypt and New Zealand. It is of minor

importance in the United States, but can be a problem in northern and eastern Europe (Harveson et al, 2009). Symptoms include reddish pustules that occur on the seed talks, petioles and both leaf surfaces (Pscheidt and Ocamb, 2018). In severe cases beet rust can cause the early senescence of leaves (Punithalingam, 1968).

Powdery mildew caused by *Erysiphe betae* was rare in the United States occurring along the Pacific Coast (Yarwood, 1937; Carsner, 1947). The first severe powdery mildew outbreak on sugar beet occurred in California in the 1970s (Coyier et al, 1975). It now occurs annually to some degree in most countries that grow sugar beet (Scott and Jaggard, 1993). When powdery mildew first appears it can be seen as small circular, white, dust-like colonies on the surface of older leaves (Francis, 2002). In the right climate and environmental conditions, powdery mildew can cover the entire leaf and can cause the death of the leaf (Bradley and Khan, 2002). Management includes the use of resistant seed and fungicides provided they are applied prior to an outbreak (Asher, 1999; Francis and Asher, 2001).

Downy mildew, caused by the oomycete *Peronospora farinose* f. sp. *betae*, occurs in almost every country in which sugar beet is grown (Choi et al, 2015). Downy mildew usually invades the young leaves at the crown of the plant causing the leaves to be stunted and malformed (Leach, 1945). In cool moist conditions hyphal growth occurs on the bottoms of the leaves, which range in color from white to grey (Pscheidt and Ocamb, 2018). This can cause affected leaves to die and may inhibit seed production (Whitney and Duffus, 1986).

Fungal root diseases cause an infection of the sugar beet taproot. These can cause a variety of symptoms including a reduction of sucrose content, root necrosis, wilting of foliage, crown rot, a reduction in the size of the taproot, and in severe cases death of the plant. Some examples of fungal root diseases include Charcoal rot (*Macrophomina phaseolina*), Rhizoctonia root rot

(*Rhizoctonia solani*), Rhizopus root rot (*Rhizopus stolonifera*), Fusarium yellows (*Fusarium oxysporum*), Verticillium wilt (*Verticillium dahlia*), Aphanomyces root rot (*Aphanomyces cochliodes*), Phytophthora root rot **(***Phytophthora drechsleri*), and damping-off (*Pythium aphanidermatum*).

Aphanomyces cochliodes is the causal agent of Aphanomyces root rot and seedling disease. It occurs in all sugar beet growing regions of the United States as well as in Canada, Europe and Japan. *A. cochliodes* causes disease under warm, wet soil conditions anytime from late June until harvest. In the seedling disease after emergence a dark grey water-soaked lesion develops on the hypocotyl, after which the entire hypocotyl turns brown to grey-black and diminishes in size (Harveson, 2013). Rot can occur in the root in seedlings but is more common in established roots. The above-ground symptoms include small non-vigorous plants that have a tendency to wilt. Below-ground the roots can develop water-soaked lesions with a tan-yellow color which results in the stunting or the root (Harveson, 2006). Management includes the use of partially resistant cultivars, seed treatment with the fungicide hymexazol, and cultural practices to reduce the spread and amount of inoculum in a field (Harveson, 2013).

There are currently more than two dozen species of nematodes that cause disease in sugar beet (Hafez, 1998). Annual yield losses due to nematodes is roughly ten percent, most of which is caused by the sugar beet cyst nematode (*Heterodera schachtii)* (Roberts and Thomason, 1981). Sugar beet cyst nematode infestation symptoms include stunted plants as well as chlorotic and wilted leaves (Gray and Gerick, 1998). In heavy sugar beet cyst nematode infestations there can be an overproduction of fibrous roots (Gray and Gerick, 1998). Some of the other nematodes that cause disease in sugar beet include the root-knot nematodes caused by *Meloidogyne* spp., false

root-knot nematodes (*Nacobbus aberans* and *N. dorsalis*), and the clover cyst nematode (*Heterodera trifolii*).

Viral diseases of sugar beet are often vectored by aphids or other insects, fungi or plasmodiophores. The most important viral disease of sugar beet is Rhizomania, which is caused by *Beet necrotic yellow vein virus* (BNYVV) (Lennefors et al, 2005; Tamada and Baba, 1973). BNYVV was first reported in the United States in 1984 (Duffus et al, 1984). Foliar symptoms of BNYVV include chlorosis and sometimes the development of necrotic yellow veins for which the virus is named (Franc et al, 1993). Root symptoms include stunting, constriction and extensive root proliferation (Rush et al, 2006). Other viral diseases of sugar beet include *Beet yellow virus*, *Beet yellow vein virus*, *Beet mosaic virus*, and *Beet curly top virus*.

Some bacteria also cause disease in sugar beet. Bacterial vascular necrosis and rot is caused by the bacteria *Pectobacterium betavasculorum*. This disease has caused considerable damage to sugar beet in portions of the United States. Bacterial vascular necrosis and rot favors warmer temperatures and symptoms include both foliar and root rot symptoms (Sahel et al, 1996). Other bacterial diseases of sugar beet include bacterial leaf spot (*Pseudomonas syringae*), Yellow wilt (phytoplasma), and Syndrome des Basses Richesses.

Cercospora

Genus

Cercospora belongs to the division Ascomycota, the class Dothideomycetes, the order Capnodiales and the family Mycosphaerellaceae. The genus *Cercospora* was created in 1863, although there is some uncertainty whether it was created by Frensious or Fuckel (Crous and Braun, 2003). The genus *Cercospora* contains many important fungal plant pathogens on a wide range of hosts. Species of *Cercospora* are commonly associated with leaf spot diseases and are

primarily host specific (Agrios, 2005). Many of these leaf spot diseases occur in agriculturally important crops such as sugar beet, maize, soybean and rice. There are more than 3,000 species described in the genus, 659 of which have been recognized (Crous and Braun, 2003). Most *Cercospora* species are known from only their morphological characteristics *in vivo* (Groenwald et al, 2012). Currently researchers such as Groenwald et al (2012) are analyzing DNA sequences derived from internal transcribed spacer regions to help further distinguish the various *Cercospora* species. As sequencing technology becomes more and more affordable, identification of *Cercospora* species will be accomplished through a combination of morpohological and genetic characteristics.

Morphology was the original method of characterizing and defining the genus *Cercospora.* One of the more reliable of the morphological characteristics was spore width since the other characteristics of conidia are influenced by environmental effects or are not distinctive (Chupp, 1954). Likewise the conidiophore structures and sizes are affected by the environment or have too much diversity within the same species.

In addition to morphology, chemotaxonomy was another older method of characterizing organisms. Chemotaxonomy is a method of identifying organisms through the use of chemicals such as secondary metabolites (Hall, 1969, 1973). One of the hallmarks of the genus *Cerocospora* is that most members of this species are able to produce the secondary metabolite cerocosporin. Cercosporin is a non-host-specific toxin, which has been associated with the virulence of species within host plants. Consequently the morphological criteria for what constitutes the genus *Cercospora* has changed many times since it was first described in 1863.

Cercospora beticola

Cercospora beticola is the causal agent of Cercospora leaf spot. Cercospora leaf spot is the most destructive foliar disease of sugar beet capable of causing losses of 40 percent or more (Jacobsen and Franc, 2009). *C. beticola* was first described by Saccardo (1876). The center of origin of *C. beticola* is central Europe and the Mediterranean (Groenewald et al, 2005). One of the earliest descriptions of Cercospora leaf spot was in 1886 by von Thümen, which he called "Blattfleckenkrankheit der Zuckerrübe" that roughly translates to sugar beet leaf stain disease. Later in 1895, Halsted gave additional descriptions of Cercospora leaf spot including the first photograph of the symptoms.

The impact of Cercospora leaf spot on sugar beet production can be severe. An example of this occurred in 1998 when 2.3 million ha of a 7 million ha sugar beet crop was lost to Cercospora leaf spot (Holtschulte, 2000). The yield losses from that year in North Dakota and Minnesota resulted in an economic loss of $113 million (Cattanach, 1999).

In addition to sugar beet, *Cercospora beticola* is able to infect table beet, celery, swiss chard, safflower, spinach and sea beet (Lartey et al, 2005;Vestal, 1933; Koike et al, 2010). *C. beticola* also has wild hosts with species in the genera *Amaranthus*, *Atriplex*, *Chenopodium*, *Cycloma* and *Plantago* (Jacobsen and Franc, 2009). The sexual cycle of *C. beticola* is currently not known (Bolton et al, 2012). Without a sexual cycle, genetic variation is likely the result of mutation, vegetative compatibility, or parasexual recombination. The disease cycle of *C. beticola* on sugar beet starts when the environmental conditions are favorable. The release of conidia requires high humidity (greater than 98% relative humidity), and temperatures between 20 and 26 °C (Vereijssen, 2004). After release, conidia can be spread a short distance by splashing water and by wind (Weiland and Koch, 2004). Once on the plant, infection takes place with the fungus

entering the plant through the stomata. When the stomata are open *C. beticola* enters via hyphae but when stomata are closed it enters via appressoria (Rathaiah, 1976). Conditions optimal for infection include temperatures between 20 and 26 °C and relative humidity greater than 98 percent for 10 to 12 hours a day for three to five days (Pool and McKay, 1916; Mischke, 1960). As a hemi-biotroph, *C. beticola* starts the infection process with an asymptomatic biotrophic phase where the pathogen grows within the leaf parenchymal tissue intercellularly (Steinkamp et al, 1979). This phase lasts approximately seven days (Feindt et al, 1981; Steinkamp, et al, 1979). After the biotrophic phase, the fungus moves into a necrotrophic stage where the pathogen secretes toxins (Weiland and Koch, 2004). Once this occurs, necrotic lesions start to become visible due to cellular death. Within the necrotic tissue, conidiophores develop and release conidia. *C. beticola* is polycyclic and thus is able to have multiple disease cycles within a growing season provided that environmental conditions are favorable (Franc, 2010). *C. beticola* primarily overwinters in infected leaves as conidia and pseudostroma. Conidia is able to survive one to four months on leaf debris while pseudostroma may survive up to two years (Pool and McKay, 1916; McKay and Pool, 1918; Canova, 1959).

Symptoms of Cercospora leaf spot are roughly circular lesions that range from two to five mm in diameter that develop on older leaves (Ruppel, 1986; Duffus and Ruppel, 1993). The lesions at the center are tan to light brown, with the borders having a dark brown to a reddish purple color (the color becoming more the reddish purple with increasing anthocyanin content). Elongated lesions can occur on petioles and circular lesions can occur on hypocotyls that are not covered by soil (Giannopolitis, 1987). Unlike many other leaf spot lesions, Cercospora leaf spot lesions do not start from the point of contact and grow outward (Weiland et al, 2004). The lesions instead appear all at once likely due to the pathogen having switched to a toxin-secreting necrotrophic stage.

(Steinkamp et al, 1979). Black fungal stroma may be speckled within mature lesions. The center of the lesions may have a grayish cast under high humidity conditions due to conidiophores and conidia on the stroma (Vereijssen, 2004). As the disease advances, individual lesions coalesce and leaves become brown and necrotic. Cercospora leaf spot decreases sugar beet yield and sucrose content of the roots by damaging the leaves which limits photosynthesis (Weltmeier et al, 2011)

Management of Cercospora leaf spot is key in reducing yield loss. The most important component of Cercospora leaf spot management is timely fungicide applications in conjunction with disease forecasting models. In the early 1900s the first chemical control for Cercospora leaf spot was inorganic copper (Meriggi et al, 2000). Current effective fungicides are limited with triazole fungicides being the most effective (Dunsmore, 2017). Other management strategies include use of resistant cultivars, a two to three year crop rotation to reduce the amount of inoculum, destruction of crop residues and planting new sugar beet crops at least 100 m from infected residues (Harveson et al, 2009).

Genetic analysis of the *C. beticola* genome has shown that it is highly enriched with secondary metabolite clusters (de Jonge et al, 2018). To date, 63 clusters have been annotated which is almost twice as many as commonly found in closely-related *Dothideomycetes* which have 34 clusters on average (de Jonge et al, 2018). The *C. beticola* genome encodes 23 candidate non-ribosomal peptide synthetase (NRPS) clusters whereas most *Dothidiomycetes* average 13 (de Jonge et al, 2018; Ohm et al, 2012). NRPSs produce non-ribosomal peptides and are independent of messenger RNA. *Cercospora beticola* is well known for production of the secondary metabolites cercosporin and beticolin. While cercosporin has been shown to be produced widely within the genus *Cercospora*, beticolins are only been found to be produced by a few species (Milat et al, 2010). Beticolins belong to the family of compounds called xanthraquinones (Milat

et al, 2010). Beticolins interact with leaf cellular membranes in several ways and may in some ways counteract plant defenses.

Cercosporin

Cercosporin is a secondary metabolite that is almost exclusively produced by members of the genus *Cercospora* (Daub,1982a; Daub et al, 2005). The fungi *Pseudocercosporella capsellae* (a pathogen of Brassicaceae) and *Colletotrichum fioriniae* (a pathogen of apple) have been confirmed to also produce cercosporin (Gunasinghe et al, 2016; de Jonge et al, 2018). Cercosporin biosynthesis genes have been found in other *Dothidiomycetes* (de Jonge et al, 2018), but it is not known if they produce this secondary metabolite. Cercosporin was first isolated in 1957 from *C. Kikuchii*, but the structure, stereochemistry and function of this molecule were not described until 1971 (Kuyama, 1957; Lousberg, et al, 1971; Yamazaki and Ogawa, 1972). Cercosporin is a perlenequinone, which is a class of secondary metabolites characterized by a core pentacyclic conjugated chromophore (Daub and Hangarter, 1983). The chromophore structure is the reason cercosporin is light activated (Daub and Hangarter, 1983). Once activated cercosporin reacts with oxygen and generates reactive oxygen species such as singlet oxygen ($^1O_2$) and superoxide (O_2^-) (Daub and Hangarter, 1983). Reactive oxygen species cause damage to the cell by lipid peroxidation of cell membranes and electrolyte leakage (Daub,1982b; Daub and Briggs, 1983; Daub and Ehrenshaft, 2000). Additionally cercosporin is a nonspecific toxin that is toxic to almost all organisms including bacteria, mammals, plants and most fungi with the exception of species that produce cercosporin, which exhibit autoresistance.

Cercosporin production is light dependent because light triggers the induction of the genes responsible for cercosporin biosynthesis (Daub and Ehrenshaft, 2000). Cercosporin was verified as a photosensitizer in the 1970s by Yamazaki et al. (1975). A study by Daub (1982b) showed that

the light wavelengths required for the death of plant cells in the presence of cercosporin were the same as those absorbed by cercosporin (Daub,1982b). This indicates that cercosporin is responsible for cell death rather than another light-responsive event.

The cercosporin biosynthetic pathway was recently studied in *Cercospora nicotianae* (Chen et al, 2007). The first gene to be found was *CTB1*, an iterative non-reducing polyketide synthase (NR-PKS) discovered through evaluation of cercosporin deficient mutants of *C. necotianae* (Choquer et al, 2005). The discovery of *CTB3* located adjacent to *CTB1* suggested that the *CTB* genes were clustered (Dekkers et al, 2007; Chen et al, 2007). Genes involved with fungal secondary metabolite synthesis have often been found in clusters, including biosynthetic genes for aflatoxins, penicillin and trichothecenes (Hohn et al., 1993; Brown et al., 1996; 2004; Brakhaage, 1998; Young et al., 2001; 2006; Abe et al., 2002; Ahn et al., 2002; Proctor et al., 2003; Gardiner et al., 2004; 2005; Yu et al., 2004; Haarmann et al., 2005; Spiering et al., 2005; Tudzynski, 2005).

Chen et al (2007) sequenced the regions adjacent to *CTB1* and *CTB3* and found a cluster of eight *CTB* genes. Six of these genes (*CTB1, 2, 3, 5, 6,* and *7*) are thought to be responsible for cercosporin assembly (Chen et al, 2007; Newman and Townsend, 2016). The zinc finger transcription factor *CTB8* functions to co-regulate the expression of the CTB cluster (Chen et al, 2007). Finally, *CTB4* is a MFS transporter that exports the completed cercosporin molecule (Choquer et al, 2007). Chen et al (2007) also noted that all of the *CTB1-8* were light regulated, while the adjacent open reading frames (*ORF11* and *ORF12*) were not. Chen et al (2007) also studied the occurrence of feedback inhibition of the *CTB1-8* genes. Feedback inhibition is the inhibition of an enzyme controlling an early stage of a series of biochemical reactions by the end product (Hubbard and Stadtman, 1966). Chen et al (2007) found that the disruption of *CTB1* partially reduced the gene expression of *CTB3* and *CTB8* and turned off gene expression

completely in *CTB2* and *CTB4*. The disruption of the *CTB2* gene inhibited *CTB1 to 4* and *CTB8* genes.

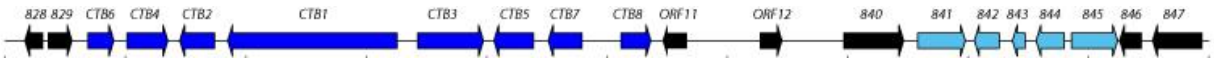

Figure 1.1. The cercosporin toxin biosynthesis (CTB) gene cluster in *Cercospora beticola*. Arrows indicate the orientation of transcription. Dark blue indicates the first cluster to be identified. Light blue indicates the more recently identified extension of the CTB cluster (de Jonge et al, 2018)

A study by de Jonge et al (2018) examined the *CTB* cluster and flanking genes in *Cercospora beticola* (*CTB1-CTB8* and *840-847*) (Fig. 1.1). They found that all but two of the genes on the 3' flank were induced by light except *CBET3_00846* and *CBET3_00848*. Functional annotation of these genes revealed that *CBET3_00841* is the cercosporin facilitator protein (*CFP*) that encodes a major facilitator superfamily (MFS) transporter that provides autoresistance (Callahan et al, 1999). *CBET3_00842* (*CTB9*) was identified as a candidate α-ketoglutarate-dependent dioxygenase responsible for the oxidative ring closure in conjunction with the dehydratase *CBET3_00843* (*CTB10*) (de Jonge et al, 2018). It is hypothesized that the βig-h3 fasciclin *CBET3_00844* (*CTB11*) and the laccase *CBET3_00845* (*CTB12*) may act early in the pathway to dimerize the product of *CTB3* (de Jonge et al, 2018). It is not known if the non-conserved phenylalanine ammonia lyase *CBET3_00840*, the zinc finger domain-containing protein *CBET3_00846* or the protein phosphatase 2A *CBET3_00847* play a role in cercosporin biosynthesis (de Jonge et al, 2018).

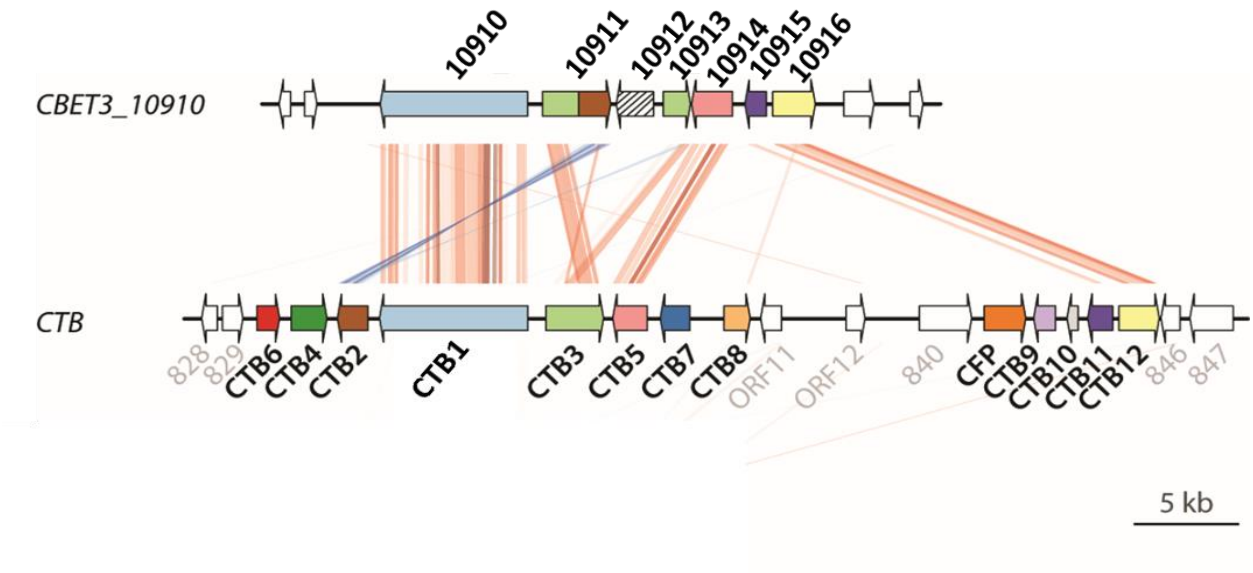

Figure 1.2. The *CTB* cluster duplication in *C. beticola*. Alignment lines correspond to DNA fragments exhibiting significant similarity when the genomic regions comprising the gene clusters are compared with tBLASTx. Direct hits are displayed in red, whereas complementary hits are in blue. The intensity of the alignments represents the percentage similarity (de Jonge et al, 2018).

While studying the evolutionary relationships of *C. beticola*, de Jonge et al (2018) found that there was significant similarity between *CbCTB1* and *CBET3_10910* as well as many of the flanking genes (Fig. 1.2). de Jonge et al (2018) hypothesized that the *CBET3_10910* cluster is the result of a CTB cluster duplication. SM cluster duplication is a fairly rare occurrence in fungi (Medema et al, 2014). Further examination of the origin and specificity of the *CTB* cluster led to the identification of *CbCTB*1 orthologs in a diverse group fungal orders including *Cladosporium fulvum*, *Colletotrichum gramincola*, and *Magnaporthe oryzae* (de Jonge et al, 2018).

The research on the CTB cluster, the expanded CTB cluster and CTB cluster duplication in *C. beticola* leads to the objectives of my research. These objectives are:

1. Determine the role *CbCTB8* plays in regulating gene expression in the expanded *CTB* cluster genes.

2. Establish whether feedback inhibition occurs in the expanded *C. beticola CTB* cluster.
3. Determine what role if any the duplicated CTB cluster plays in cercosporin production.
4. Ascertain if the duplicated CTB cluster is activated by light.

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CHAPTER 2. EXPRESSION ANALYSIS OF THE EXPANDED CERCOSPORIN GENE CLUSTER IN *CERCOSPORA BETICOLA*

Abstract

Cercospora beticola is the cause of the most economically damaging disease of sugar beet, Cercospora leaf spot. *Cercospora* species produce the photoactivated toxin cercosporin, which has broad-spectrum toxicity. Studies have shown that the genes coding for biosynthesis of cercosporin are clustered, known as the cercosporin toxin biosynthesis (*CTB*) cluster. One of the genes in the *CTB* cluster is the zinc finger transcription factor *CTB8*, which in *C. nicotianae* has exhibited feedback inhibition within the *CTB* cluster. Additional *CTB* genes were recently identified adjacent to the core *CTB* cluster. We show through qPCR analysis that *CTB8* in *C. beticola* exhibits feedback inhibition for both the core *CTB* cluster and the recently identified *CTB* genes. Further analysis into the recently identified *CTB* genes has shown that feedback inhibition occurs in some of the recently identified *CTB* genes. In addition to the recently identified *CTB* genes, a duplication of the *CTB* cluster was identified. We demonstrated that unlike the *CTB* cluster, the duplicated cluster is not light activated.

Introduction

Many species within the genus *Cercospora* produce the secondary metabolite toxin cercosporin. This toxin is considered a virulence factor because it is required for the pathogen to reach high levels of virulence (Daub, 1982a; Daub et al, 2005). Cercosporin is toxic because it reacts with light to generate reactive oxygen species that cause peroxidation of cellular membranes and electrolyte leakage (Daub and Hangarter, 1983; Daub, 1982b; Daub and Briggs, 1983).

While there has been a considerable amount of research on cercosporin toxicity and biology, the genes underlying cercosporin biosynthesis have only recently been resolved. *C.*

nicotianae was originally determined to consist of eight contiguous genes involved with cercosporin biosynthesis called the cercosporin toxin biosynthesis (*CTB*) cluster (Chen et al, 2007). The *CTB* cluster consisted of six genes responsible for cercosporin assembly, a zinc finger transcription factor (*CTB8*) and a major facilitator superfamily transporter (*CTB4*) (Chen et al, 2007; Newman and Townsend, 2016). On one side of the *CTB* cluster are two open reading frames, which unlike the *CTB* cluster, were not light activated and not believed to be involved with metabolic functions (Chen et al, 2007). This led researchers to believe the *CTB* cluster only consisted of eight genes although these have not fully explained cercosporin biosynthesis (Newman and Townsend, 2016).

Building on Chen et al (2007), a study by de Jonge et al (2018) examined the *CTB* cluster and flanking genes in *Cercospora beticola* (*CTB1-CTB8* and *840-847*) (Fig. 2.1). All but two of the genes on the 3' flank were induced by light except *846* and *848*. Functional annotation of these genes revealed that *841* is the cercosporin facilitator protein (*CFP*) that encodes a major facilitator superfamily (MFS) transporter providing cercosporin autoresistance (Callahan et al, 1999). *842* (*CTB9*) was identified as a candidate α-ketoglutarate-dependent dioxygenase responsible for the oxidative ring closure in conjunction with the dehydratase *843* (*CTB10*) (de Jonge et al, 2018). It is hypothesized that βig-h3 fasciclin *844* (*CTB11*) and laccase *845* (*CTB12*) may act early in the pathway to dimerize the product of *CTB3* (de Jonge et al, 2018). It is not known if the non-conserved phenylalanine ammonia lyase (*840*), the zinc finger domain-containing protein (*846*) or the protein phosphatase 2A (*847*) play a role in cercosporin biosynthesis (de Jonge et al, 2018).

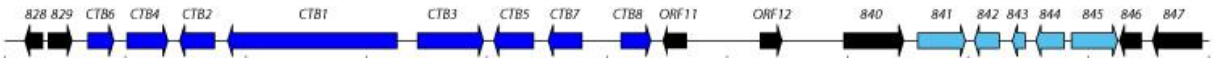

Figure 2.1. The cercosporin toxin biosynthesis (*CTB*) gene cluster in *Cercospora beticola*. Transcription map of the *CTB* genes and adjacent genes. Arrows indicate the orientation of transcription. Dark blue indicates the first cluster to be identified. Light blue indicates the more recently identified *CTB* genes (de Jonge et al, 2018)

de Jonge et al (2018) found that there is a significant similarity between the *CTB* cluster and *CBET3_10910* through *CBET3_10916* (Fig. 2.2). They hypothesized that the *CBET3_10910* through *CBET3_10916* cluster is the result of cluster duplication. Secondary metabolite cluster duplication is a fairly rare occurrence in fungi (Medema et al, 2014).

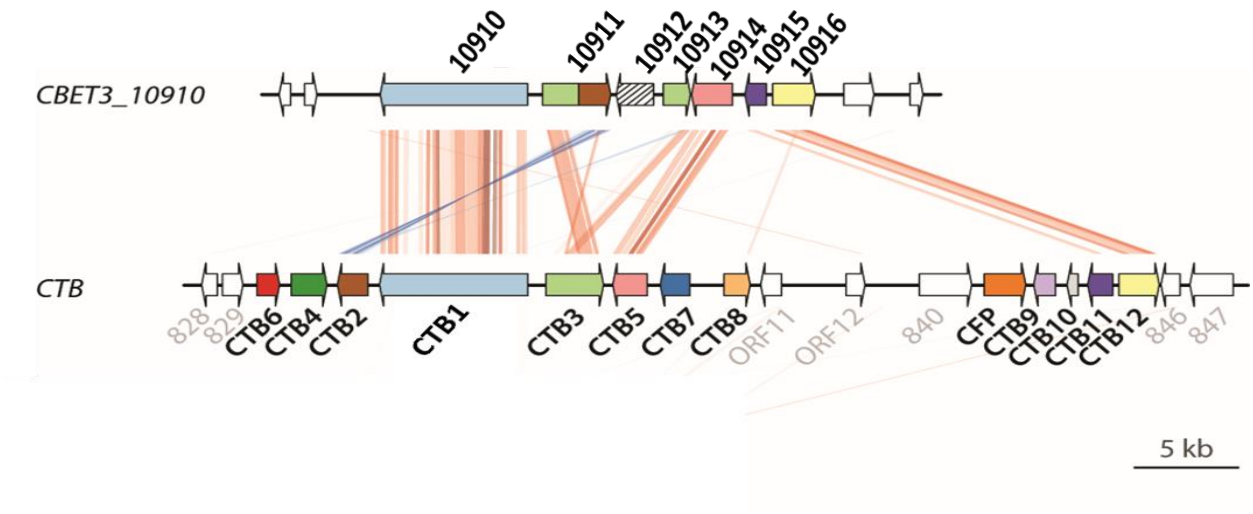

Figure 2.2. The *CTB* cluster duplication in *C. beticola*. Alignment lines correspond to DNA fragments exhibiting significant similarity when the genomic regions comprising the gene clusters are compared with tBLASTx. Direct hits are displayed in red, whereas complementary hits are in blue. The intensity of the alignments represents the percentage similarity (de Jonge et al, 2018).

Previous studies in *C. nicotiane* have shown the zinc finger transcription factor (*CTB8*) null mutant nearly abolishes the expression of the *CTB1-CTB7* genes (Chen et al, 2007). This indicates that *CTB8* controls cercosporin production by controlling gene transcript levels (Chen et al, 2007). In this paper we show that this holds true in *C. beticola* and for the newly identified *CTB* genes through qPCR analysis of a *CbCTB8* mutant. Chen et al (2007) identified that feedback inhibition occurs in the CTB cluster but is not absolutely stringent. Thus null mutants of the *C. beticola* genes 840 through 846 and 841-845 were analyzed using qPCR to determine gene regulation. It is well known that cercosporin production is light activated (Daub and Ehrenshaft,

2000). So far all of the genes involved with cercosporin toxin biosynthesis are shown to be upregulated in the presence of light (de Jonge et al, 2018; Chen et al, 2007). Here we demonstrate that unlike the *CTB* cluster, the duplicated cluster is not light activated. These results suggest that the duplicated cluster does not play a role in cercosporin production. This work provides a better understanding of gene regulation on an important toxin.

Materials and Methods

Fungal Strains and Culture Maintenance

Cercospora beticola wild-type (WT) (10-73-4 and 1-90) and genetically modified strains were maintained on potato dextrose agar (PDA; Difco). Cultures were maintained under natural light at 21° C. The fungal culture used for targeted gene replacement was the WT strain 10-73-4. The Δ*840*, Δ*841*, Δ*842*, Δ*843*, Δ*844*, Δ*845*, Δ*846*, Δ*841-845*, and Δ*10910* mutants were created in a previous study (de Jonge et al, 2017).

Split-marker Targeted Gene Replacement

Split-marker PCR constructs for targeted gene replacement were prepared as described by Catlett et al. (2003) using genomic DNA of 10-73-4 WT *C. beticola* as the PCR template. Briefly, PCR was conducted to amplify two fragments HY and YG, which represent the first two thirds (HY) and the last two thirds (YG) of the hygromycin-resistance gene (*hph*) from pDAN (Friesen et al. 2006) using M13F/HY and M13R/YG primers, respectively. PCR reactions (25µl) were conducted using GoTaq Flexi DNA Polymerase (Promega Corp., Madison, WI) kit following the manufacturer's protocol. The PCR reaction was carried out using a PTC-200 thermal cycler (MJ Research, Hercules, CA) using the following cycling conditions: 94 °C initial denaturation step for 3 minutes, followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 55 °C and 30 seconds, extension at 72 °C for 1 minute, and then one final extension step at 72 °C

extension for 5 minutes. 5’ and 3’ flanking region of the targeted gene were amplified using the primers 1F/2R and 3F/4R of each gene, respectively using PCR conditions described above. Two constructions (5’ construct and 3’ construct) were developed by fusing the two marker fragments (HY and YG) with 5’ flanking region and 3’ flanking region of target gene using 1F/HY and YG/4R primers respectively through fusion PCR. Fusion PCR reactions (50µl) contained 50 ng of each fragment, 1x reaction buffer, 1.25 mM $MgCl_2$, 15 mM dNTP, 10 mM of each primer, and 2.5 U of Taq DNA polymerase (Promega, Madison, WI). The PCR reaction was carried out using a PTC-200 thermal cycler using the following cycling conditions: 94 °C initial denaturation for 4 minutes, followed by 42 cycles of denaturation at 94 °C for 30 seconds), 30 seconds of annealing at 60 °C, extension at 68 °C for 2 minutes, and one final cycle of extension at 68 °C for 10 minutes. Equal amount of 5’ and 3’ constructs were then mixed together and purified by ethanol precipitate for PEG-mediated transformation. Fungal protoplasts were prepared and transformed using previously described methods by Bolton et al (2016). Hyg-resistant colonies were screened to confirm *CbCTB8* disruption using PCR. A primer set including 5’1F and HygR marker sequence, as well as the qPCR primer sets (Table. S1) were used to confirm disruption.

RNA Extraction

For RNA extraction, strains were grown on 60 mm diameter round plastic Petri dishes filled with thin PDA (3 ml) to induce the production of cercosporin. Cultures were initiated with a 5 mm diameter mycelial plug taken from the periphery of the source culture. All source cultures were grown to roughly the same circumference (between 3.5 and 4.5 cm) before transfer. The plates were arranged into a randomized complete block design with three reps. Plates were left to grow for 14 days in either natural light or complete darkness (achieved by wrapping plates in a double layer of aluminum foil). Initially plates were grown with the agar side of the plate being

on the bottom. After two days the plates were turned over so the agar side of the plate was on the top.

Total RNA was extracted from fungal tissue frozen in liquid nitrogen using RNeasy Plant Mini Kit reagents (Qiagen) following the manufacturer's protocol with some modifications. To obtain purified RNA, the optional step of DNase Digestion of RNA before RNA Cleanup was performed. An additional DNase treatment was performed using RQ1 RNase-Free DNase (Promega, Madison, WI) according to the manufacturer's recommendations.

Quantitative RT-PCR Analysis

Synthesis of cDNA was carried out using SuperScript IV First-Strand cDNA Synthesis Reaction reagents (Thermo Fisher, Minneapolis, MN) according to the manufacturer's recommendations. GoTaq qPCR Master Mix (Promega, Madison, WI) was used for q-RT PCR reactions. The primers used to amplify each gene are shown in Table 2.1. The qPCR was performed using a CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The reaction conditions were set as follows: 95°C denaturation step for 30 seconds followed by annealing at 60°C for 30 seconds, and extension at 72°C for 45 seconds. The q-RT PCR reactions were performed in triplicate, and negative controls included: i) the use of RNA as a template to check for gDNA contamination in samples, and ii) a water control. Each sample was normalized against the actin control, and fold-change relative to WT was calculated according to the Pfaffl method (Pfaffl, 2001).

Table 2.1. Primer sequences used in this study.

Gene	Forward Primer	Reverse Primer
Split-marker Primers		
Hygromycin Yg-F	CGTTGCAAGACCTGCCTGAA	
Hygromycin Hy-R		GGATGCCTCCGCTCGAAGTA
CTB1 deletion Primers		
CTB1-1F and CTB1-2R	TCCTCTGGTGCTATGTCACG	CACTGGCCGTCGTTTTACAACGT CGAGATGGCAGAGGTACAGCT

Table 2.1. Primer sequences used in this study (continued).

Gene	Forward Primer	Reverse Primer
CTB1 deletion Primers		
CTB1-3F and CTB1-4R	TCATGGTCATAGCTGTTTCCTGT GTAACTCCGTCTCCAACCACC	CTGGTCGAGAAACTTGTGCA
CTB1 5'1F	GAGCGTGCTGTTTCCCTATG	
CTB8 deletion Primers		
CTB8-1F and CTB8-2R	CGCACATGTGGAATAAGTGG	CACTGGCCGTCGTTTTACAACGT CGGTTGAAAGCCTATCGGACA
CTB8-3F and CTB8-4R	TCATGGTCATAGCTGTTTCCTGT GTCATGCTTGGTGCTGACTTC	TCCATGTATGGGGCGATAGT
CTB8 5'1F	CGCCTACTGAGTACGGAAGC	
qPCR Primers		
CbCTB_00830	CGACTGGGAACACGACTTCA	CATTTCCGGAATGCGCGATT
CbCTB_00831	GTGGATCGCGAATGTCGTTG	AAGCCCAGTTGCGTGTCATA
CbCTB_00832	AAGCAGTCGGAGATGGTGTG	TCCAGGCTTCGAGATCTGGA
CbCTB_00833	AGATCGGGATGCCAATCGAC	CAATCTCCATGAACTGCGCG
CbCTB_00834	TCGCTCGCGATGAAAGCTAT	ATTCCCAGATTGAACGCGGT
CbCTB_00835	ACCGTTCCTCAAGACCGAAC	AATTGCCAGCGTTGAGGGTA
CbCTB_00836	ACTGCGATCATTGGTGCAGA	GCGACCTTTGTTGGCATGTT
CbCTB_00837	CTGCAGCGAATATCGTCCCT	TCTTGGCCACAACATCCTCC
CbCTB_00840	GCCAATGCATCACGGCTTAG	CCAAGCTCATGCATCGCTTC
CbCTB_00841	CTGGGATCCCTGGCATGTTT	GACAGCGAAGACCACAAGGA
CbCTB_00842	CTTGGTAGGATTGATCGACGTG	ACTGGAGCGACTAAGGTTCA
CbCTB_00843	GTCACCAAGAAGCCAGACCA	ACATTCTCTTCTGGCTGGCC
CbCTB_00844	GTGGCTCGCGAGGATTTTC	GCCATGAATTTCTGTGAGGCA
CbCTB_00845	AGAGAAGGCCAGACACGTTG	ACAAACGACACCATCTCGCT
CbCTB_00846	GAAGAAGGAGGTCACGGGTG	CTTCACTCCGGCCTTGTCAT
CbCTB_00847	ACGACGGCCGATACATTCTC	TGACATTCTTGGCGTCTCCC
CbCTB_00848	AAAGCCCTGTTACCAGTCCG	TACCGTCTCTCATCCTGCCA
CbCTB_010910	AGCATAACCGAGTGGGTGTC	AGTTCAAACGGCCTGGTATG
CbCTB_010911	AGTGGAGTGGTCACCAAAGG	CTTGCGTAGATGGCTGTTGA
CbCTB_010912	GTTTCTGGCATGGGTGAGAT	CGCATGACTTGATGTGCTCT
CbCTB_010913	TTCAGCCTTCTCGATTTCGT	GCTCCTTTCACAGGCTGTTC
CbCTB_010914	GAAGAAACGCTGGACTTTCG	TGTAGGGATGGGCTGGTAAG
CbCTB_010916	TGTTCAAGCCTCCGCTAGTT	GACGAGGATACCTGGACGAG
C. beticola Actin	ACATGGCTGGTCGTGATTTG	TGTCCGTCAGGAAGCTCGTA

Results

Objective 1

As mentioned previously, a null mutant of the zinc finger transcription factor (*CTB8*) in *C. nicotianae* nearly abolishes the expression of *CTB1-CTB7* genes (Chen et al, 2007). To determine the role *CbCTB8* plays in regulating gene expression in the *CTB* cluster, a *CbCTB8* disruption mutant was generated using the split marker strategy. A primer set including 5'1F and HygR marker sequence, as well as qPCR primer sets (Table 2.1) were used to confirm disruption (Fig. 2.3). The 5'1F and HygR primers confirm the knockout by having a band present for the gene of interest. The qPCR primers confirm the knockout through the absence of the gene of interest.

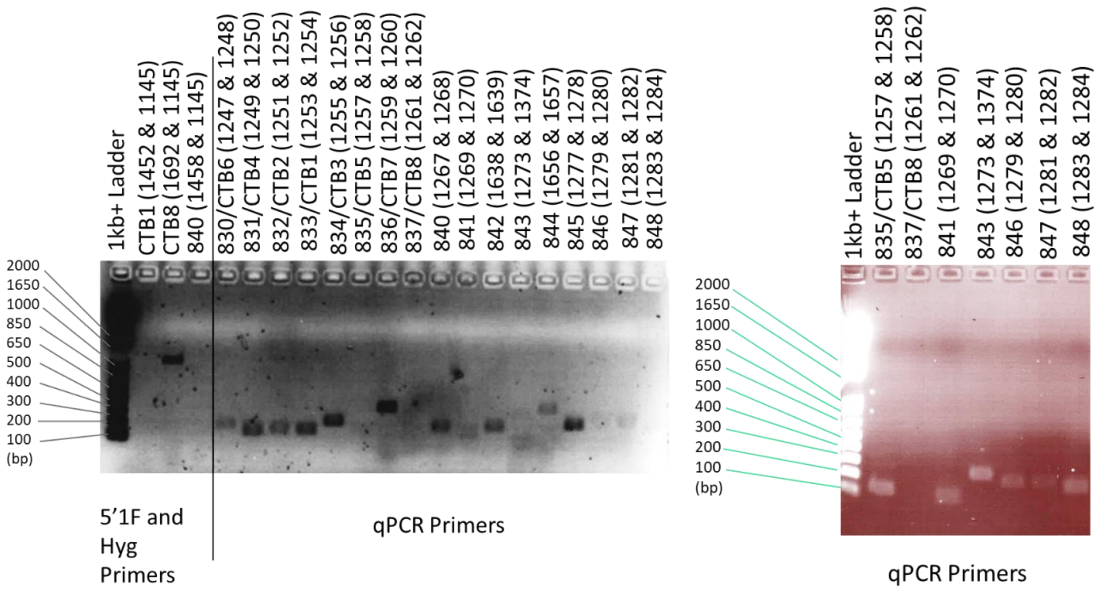

Figure 2.3. PCRs confirming Δ*CbCTB8*

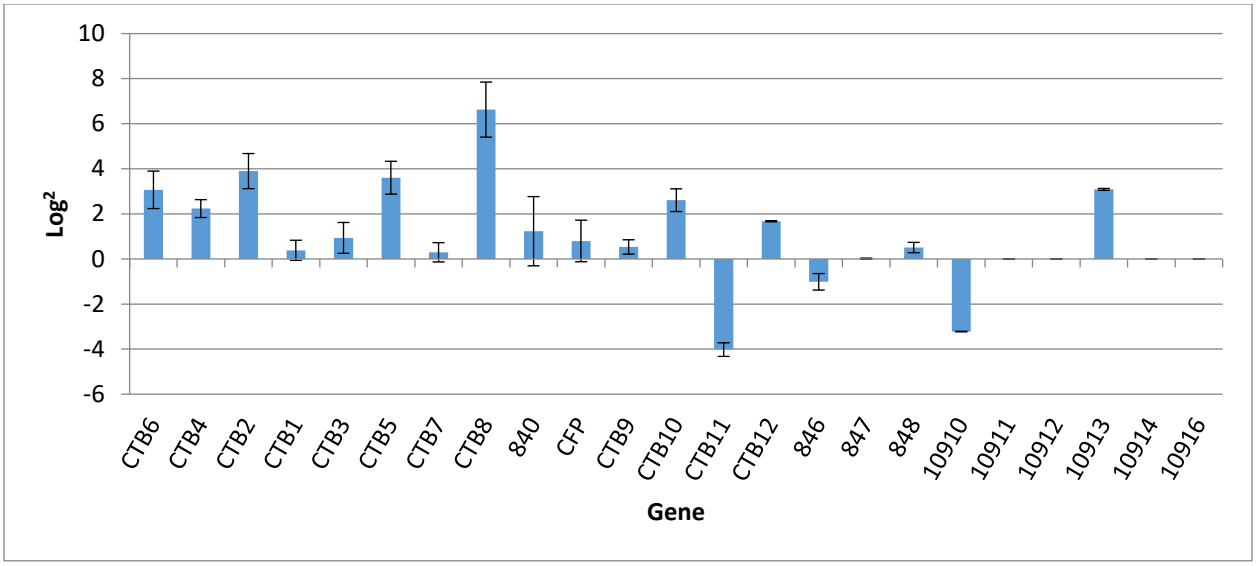

Figure 2.4. Quantitative RT-PCR analysis of WT (1-90) grown in light and WT (1-90) dark as the control. Each sample was normalized against actin control. Data represents the combination of three biological reps, each with three technical reps. Log^2 was used to model the proportional change. Error bars represent standard error.

qPCR analysis of WT cDNA showed in general most *CTB* genes were induced in the light (Fig. 2.4). *CTB8* was the most highly expressed in light followed by *CTB2, CTB5, CTB6* and *Cb10913*. In contrast *CTB11*, Cb846, and Cb10910 were all repressed in the light.

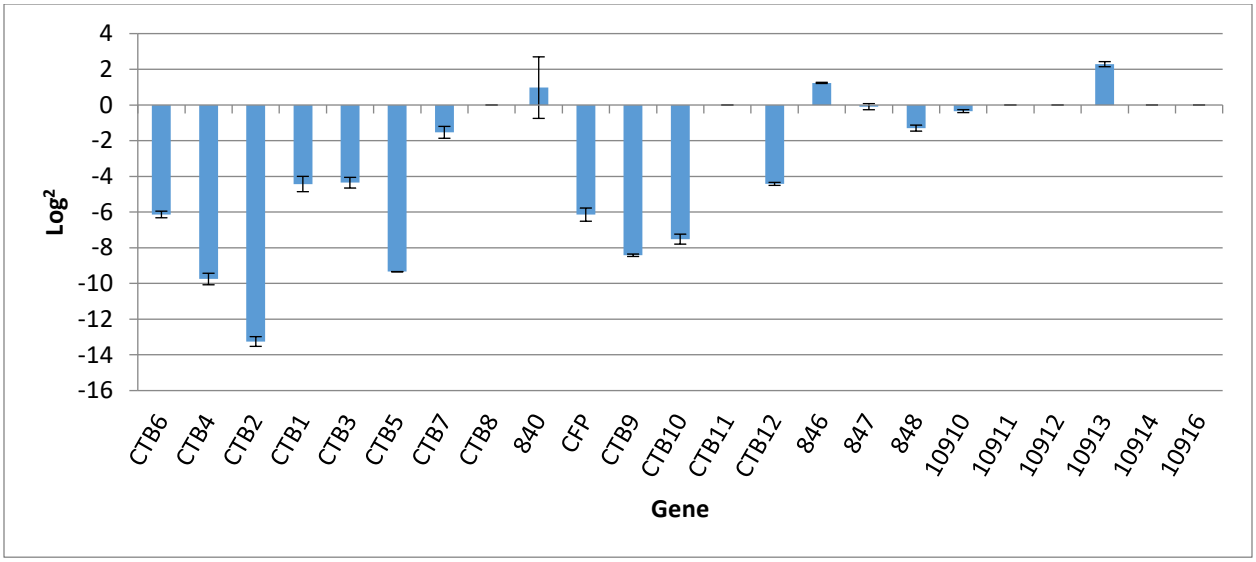

Figure 2.5. Quantitative RT-PCR analysis of **Δ***CbCTB8* grown in light using WT grown in light as control. Each sample was normalized against actin control. Data represents the combination of three biological reps, each with three technical reps. Log^2 was used to model the proportional change. Error bars represent standard error.

qPCR analysis of Δ*CbCTB8* showed all of the genes in the original cluster (*CbCTB1-7*) and the recently identified *CTB* genes (*CbCFP*, and *CbCTB9-12*) were repressed (Fig. 2.5). There was no distinguishable difference between Δ*CbCTB8* and WT in *840, 847*, and *010910*. There was little to no expression of the duplicated *CTB* cluster genes *10911* to *10916* in either Δ*CbCTB8* or WT, except for *Cb10913* which was upregulated in Δ*CbCTB8*.

Objective 2

To determine whether feedback inhibition occurs in the *CTB* expanded cluster, mutants of *CBET3_00840* to *CBET3_00846* were utilized. All of the mutants were confirmed a using primer sets including the corresponding gene 5'1F and HygR to confirm the presence of the marker as well as qPCR primer sets to confirm the absence of the targeted gene (Table 2.1). Both the WT and Δ*CBET3_00840* to Δ*CBET3_00846* were grown on thin PDA in light and expression of cluster genes was analyzed using qPCR.

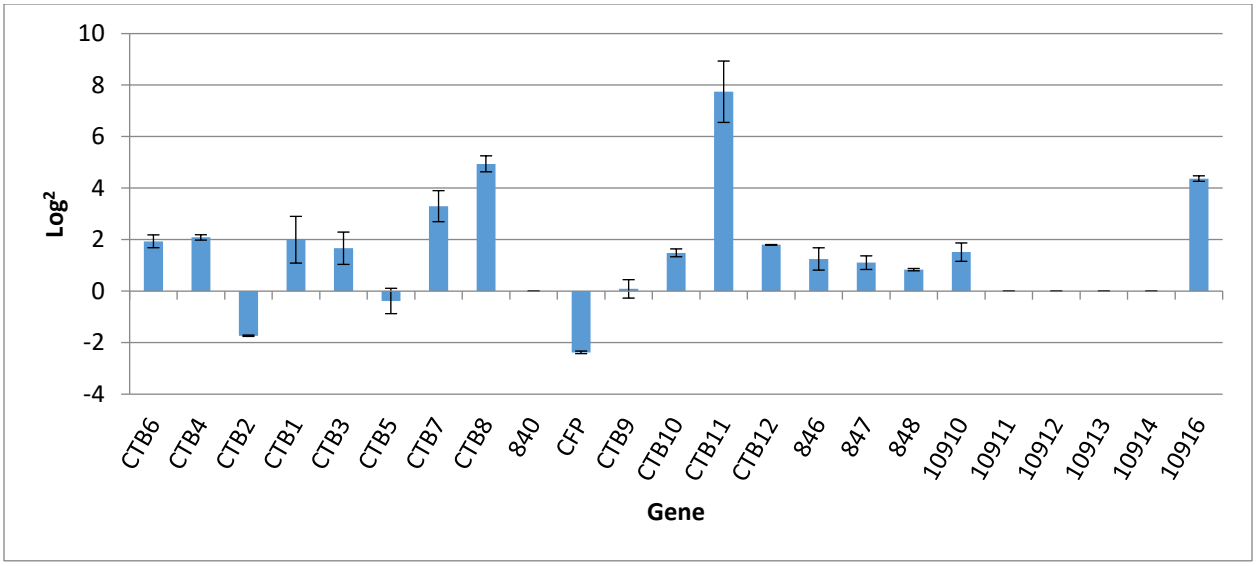

Figure 2.6. Quantitative RT-PCR analysis of Δ*Cb840* grown in light using WT grown in light as control. Each sample was normalized against actin control. Data represents the combination of three biological reps, each with three technical reps. Log^2 was used to model the proportional change. Error bars represent standard error.

CbCTB2 and *CbCFP* were significantly repressed in Δ*Cb840* (Fig. 2.6). There was no significant difference between the WT and Δ*Cb840* in *CbCTB9, Cb10911, Cb10912, Cb10913*, and *Cb10914*. There was significantly higher expression in the remaining genes compared to the WT. *CbCTB11*, *CbCTB8* and *Cb10916* were the most highly expressed in Δ*Cb840*.

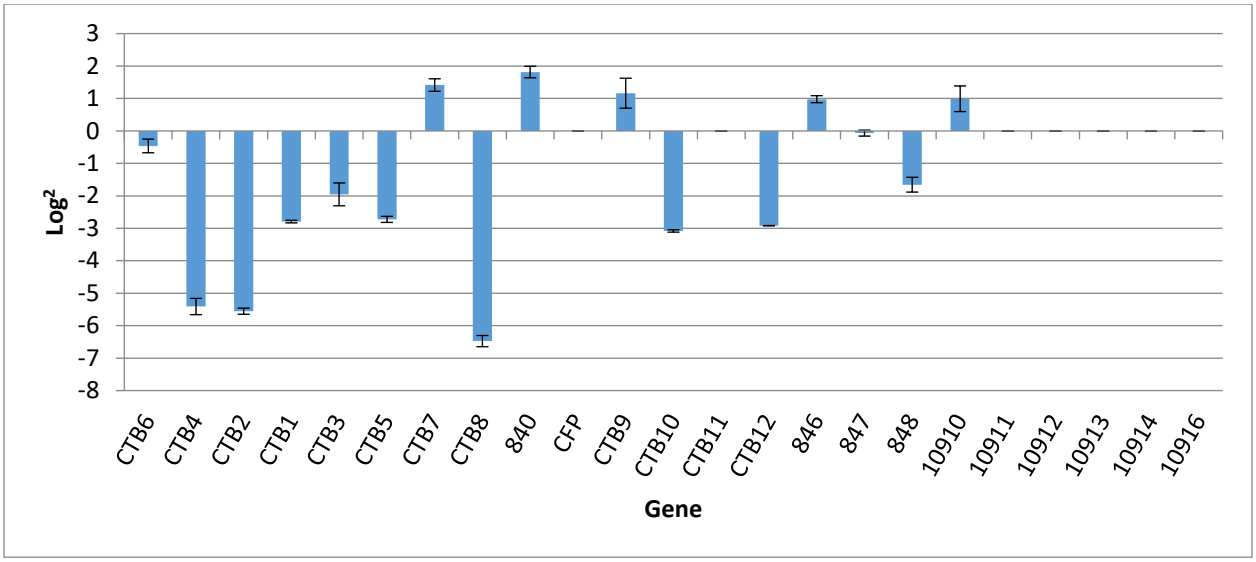

Figure 2.7. Quantitative RT-PCR analysis of Δ*CbCFP* grown in light using WT grown in light as control. Each sample was normalized against actin control. Data represents the combination of three biological reps, each with three technical reps. Log^2 was used to model the proportional change. Error bars represent standard error.

Almost all of the genes in the original cluster (*CbCTB1-8*) were repressed in Δ*CbCFP* compared to WT except *CbCTB7*, which was slightly upregulated (Fig.2.7). In the recently identified CTB genes and adjacent genes there was suppression in *CbCTB10*, *CbCTB12* and *Cb848*. *Cb840, CbCTB9, Cb846* and *Cb10910* were all slightly upregulated in Δ*CbCFP*. There was no major difference between Δ*CbCFP* and WT in *CbCTB11* and *Cb847*.

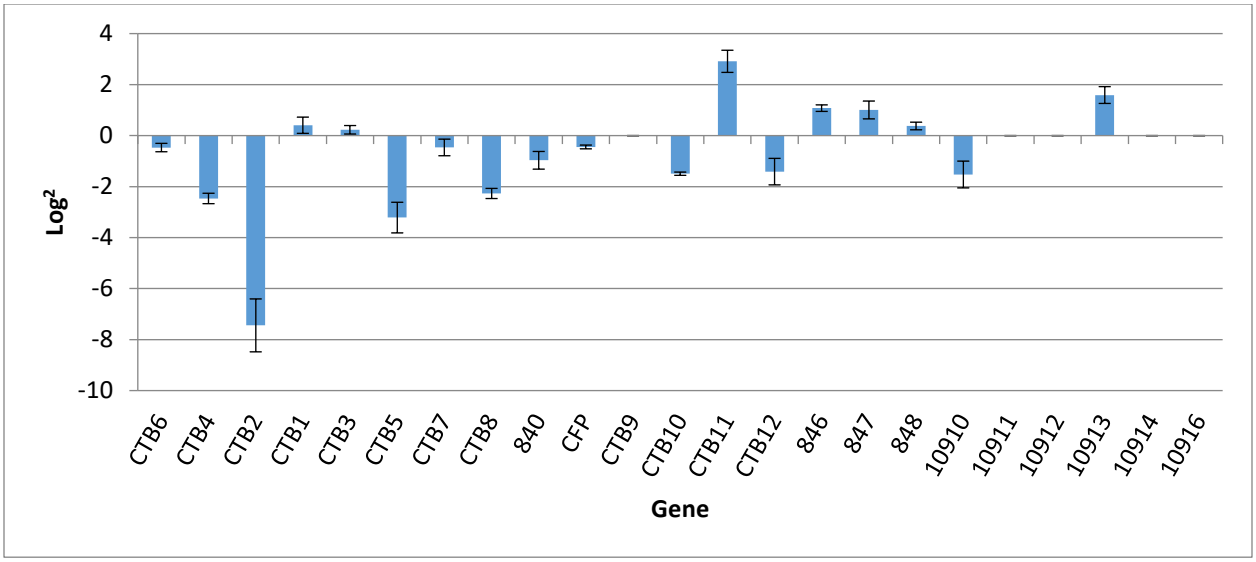

Figure 2.8. Quantitative RT-PCR analysis of Δ*CbCTB9* grown in light using WT grown in light as control. Each sample was normalized against actin control. Data represents the combination of three biological reps, each with three technical reps. Log^2 was used to model the proportional change. Error bars represent standard error.

Δ*CbCTB9* had several genes significantly repressed compared to the WT, these genes were *CbCTB4*, *CbCTB2*, *CbCTB5*, and *CbCTB8* (Fig. 2.8). *CbCTB2* was the most highly repressed in Δ*CbCTB9*. Only *CbCTB11* had significantly more expression than the WT. The remaining genes had no significant differences in expression between Δ*CbCTB9* and WT.

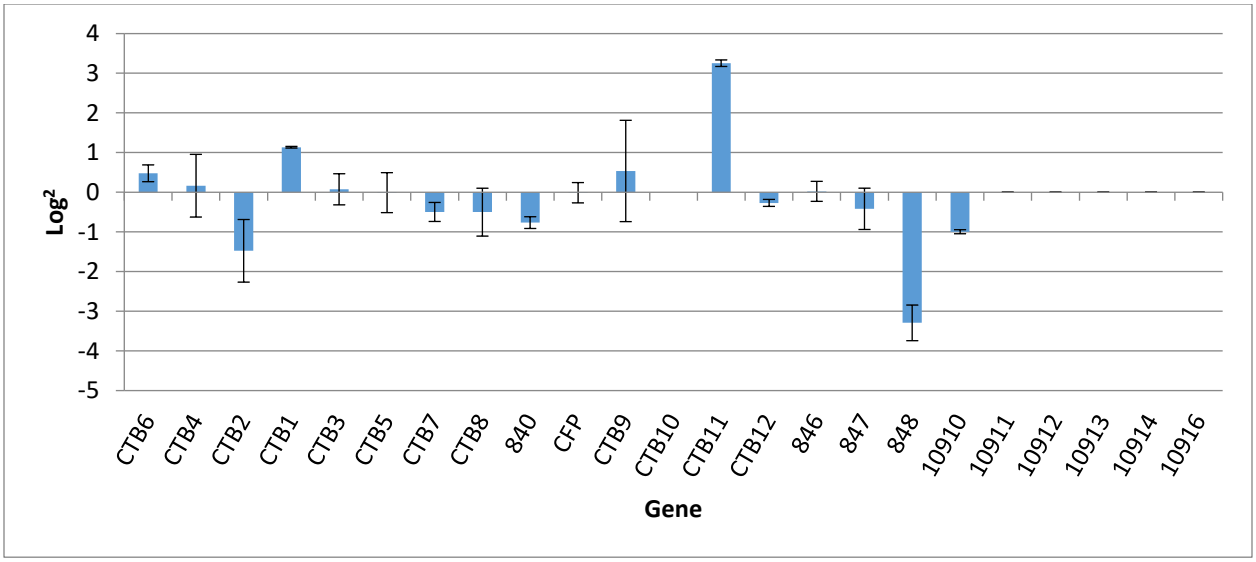

Figure 2.9. Quantitative RT-PCR analysis of Δ*CbCTB10* grown in light using WT grown in light as control. Each sample was normalized against actin control. Data represents the combination of three biological reps, each with three technical reps. Log^2 was used to model the proportional change. Error bars represent standard error.

Most of the genes in Δ*CbCTB10* were not significantly different in expression than the WT (Fig. 2.9). Only *CbCTB2* and *Cb848* were significantly repressed in Δ*CbCTB10* compared to WT and *CbCTB11* was the only gene more strongly expressed in Δ*CbCTB10* than in WT.

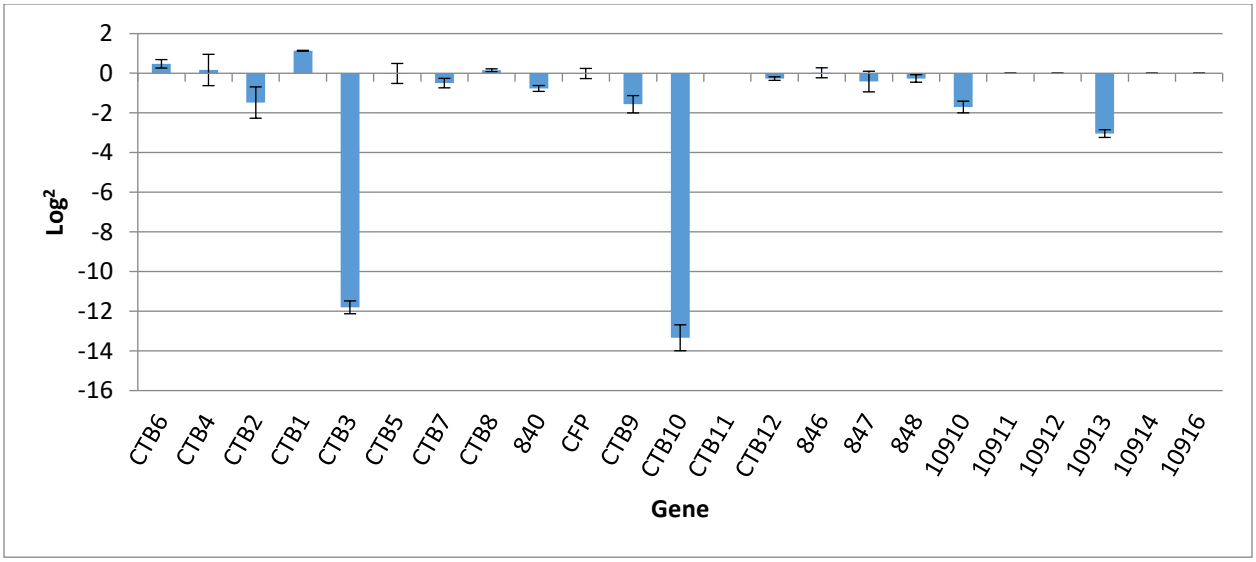

Figure 2.10. Quantitative RT-PCR analysis of **Δ***CbCTB11* grown in light using WT grown in light as control. Each sample was normalized against actin control. Data represents the combination of three biological reps, each with three technical reps. Log^2 was used to model the proportional change. Error bars represent standard error.

CbCTB3 and *CbCTB10* were dramatically repressed in Δ*CbCTB11* compared to WT (Fig. 2.10). *CbCTB2*, *CbCTB9*, *Cb10910* and *Cb10913* were also repressed to some extent in Δ*CbCTB11*. There was little to no difference in expression in the remaining genes compared to WT.

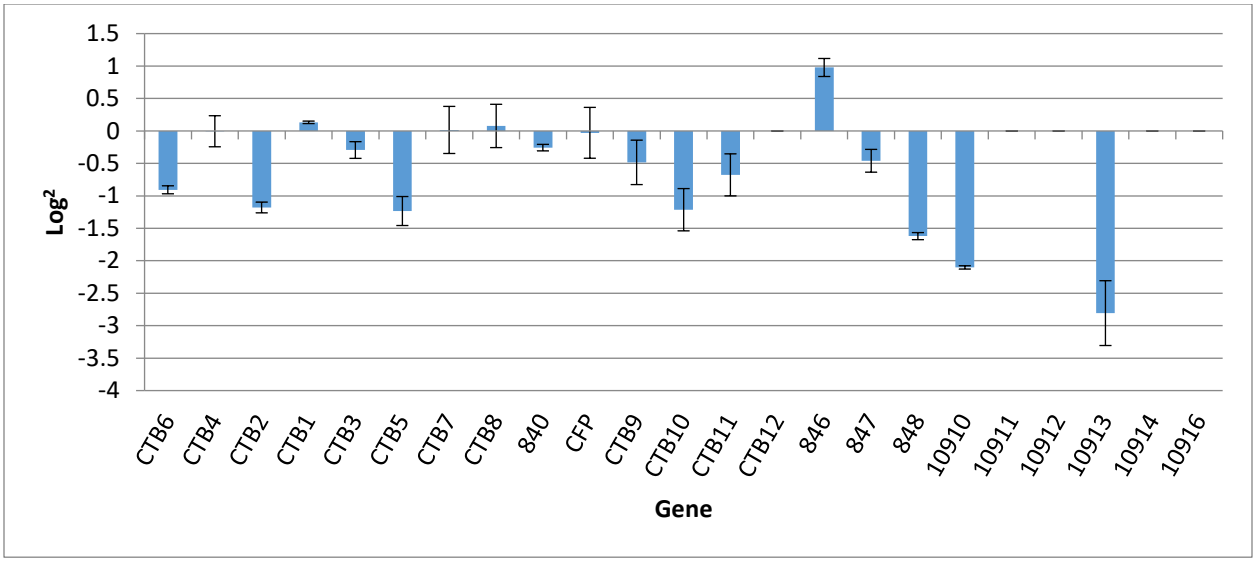

Figure 2.11. Quantitative RT-PCR analysis of **Δ***CbCTB12* grown in light using WT grown in light as control. Each sample was normalized against actin control. Data represents the combination of three biological reps, each with three technical reps. Log^2 was used to model the proportional change. Error bars represent standard error.

CbCTB2, *CbCTB5*, *CbCTB10*, *Cb848*, *Cb10910* and *Cb10913* were repressed in Δ*CbCTB12* compared to WT (Fig. 2.11). In contrast *Cb846* was more highly expressed in Δ*CbCTB12* than the WT. The remaining genes did not significantly differ from the WT.

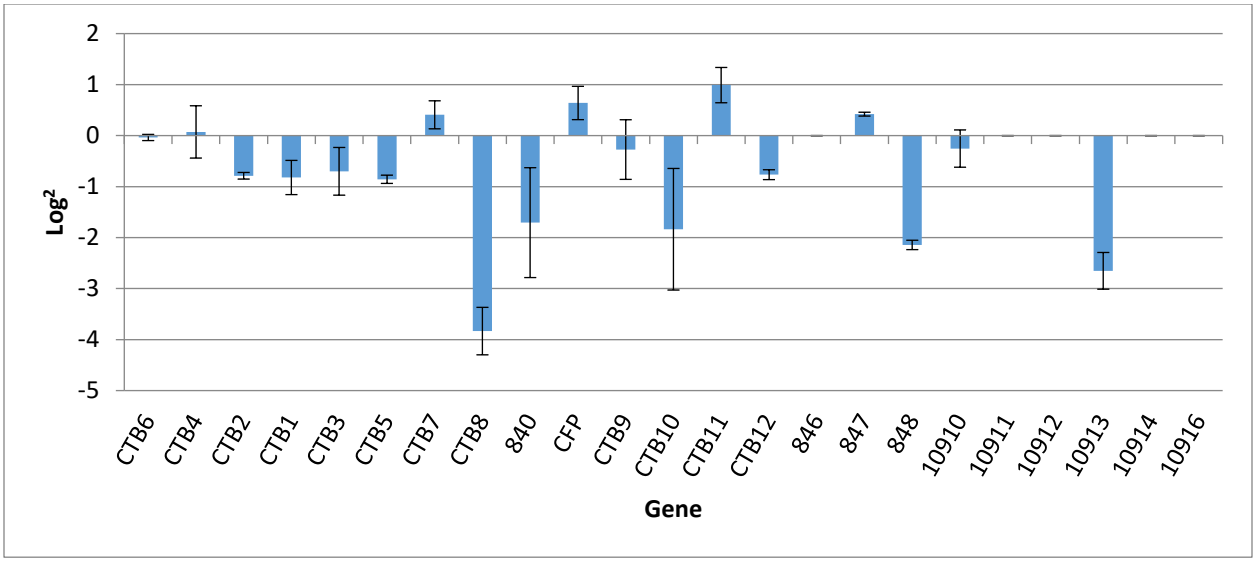

Figure 2.12. Quantitative RT-PCR analysis of **Δ***Cb846* grown in light using WT grown in light as control. Each sample was normalized against actin control. Data represents the combination of three biological reps, each with three technical reps. Log^2 was used to model the proportional change. Error bars represent standard error.

CbCTB2, *CbCTB1*, *CbCTB3*, *CbCTB5*, and *CbCTB12* were slightly repressed in Δ*Cb846* compared to WT (Fig. 2.12). *CbCTB8*, *Cb840*, *CbCTB10*, *Cb848*, and *Cb10913* were all significantly repressed in Δ*Cb846*. *CbCTB11* was more highly expressed in Δ*Cb846* than the WT. In the remaining genes there were no significant differences in gene expression between Δ*Cb846* and the WT.

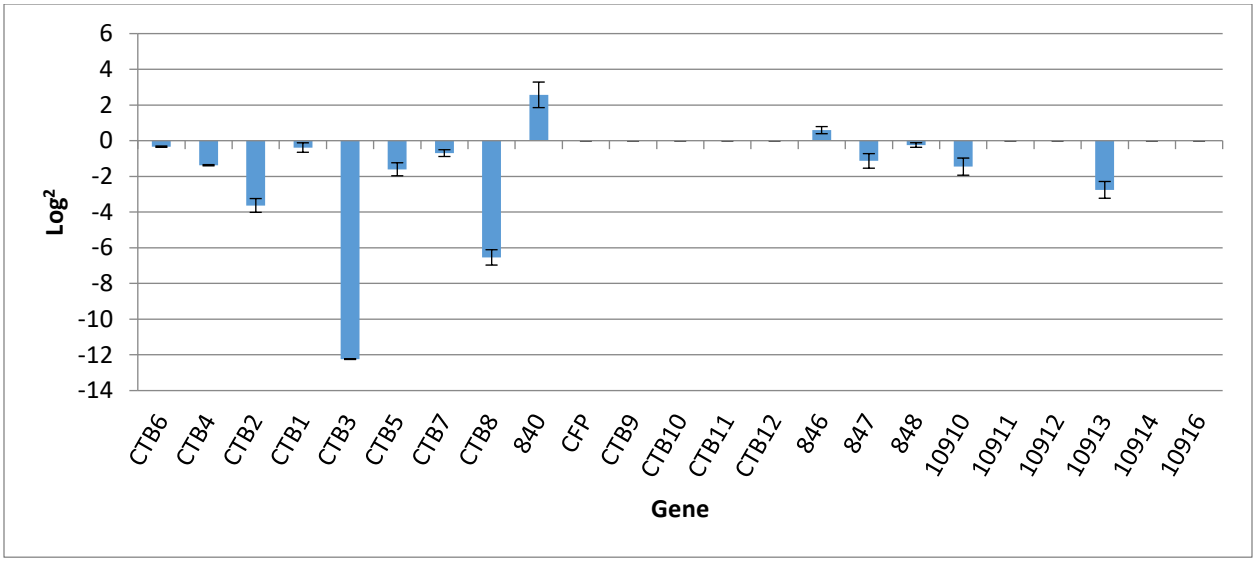

Figure 2.13. Quantitative RT-PCR analysis of Δ*Cb841-845* (*CbCFP*, *CbCTB9* to *CbCTB12*) gene mutant grown in light using WT grown in light as control. Data represents the combination of three biological reps, each with three technical reps. Log^2 was used to model the proportional change. Error bars represent standard error.

Majority of the genes were repressed in Δ*Cb841-845* (Fig. 2.13). *CbCTB3* was the most significantly repressed followed by *CbCTB8*. Expression in *CbCTB6*, *CbCTB1*, *CbCTB7*, *Cb846*, and *Cb848* was not significantly different from the WT. Cb840 was the only gene to be more highly expressed in Δ*Cb841-845* than in the WT.

Objective 3

To determine whether the duplicated cluster plays a role in cercosporin production, a *CbCTB1* and *Cb10910* mutant were generated in a previous experiment using the split marker strategy (de Jonge et al, 2018). Since *Cb10910* is a duplication of *CbCTB1*, both mutants were used as comparisons. Hyg-resistant colonies were screened to confirm *CbCTB1* disruption using PCR. Primer sets including 5'1F and HygR marker sequence and the qPCR primer sets (Table 2.1), were used to confirm disruption in both *ΔCb10910* and Δ*CbCTB1* (Fig. 2.14). The 5'1F and HygR primers confirm the knockout by having a band present for the gene of interest. The qPCR primers confirm the knockout through the absence of the gene of interest.

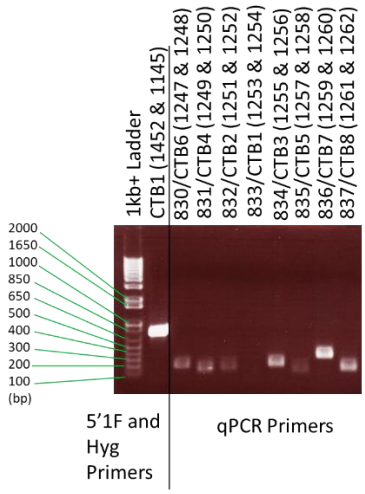

Figure 2.14. PCR confirming Δ*CbCTB1*

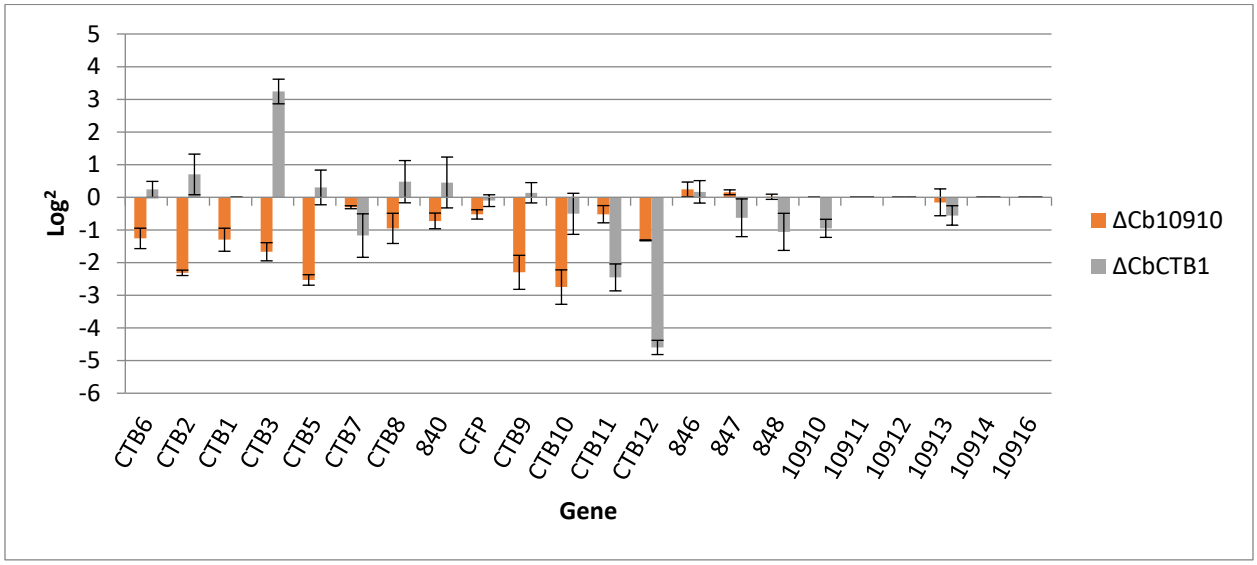

Figure 2.15. Quantitative RT-PCR analysis of **Δ***Cb10910* grown in light using WT grown in light as control. qPCR analysis of **Δ***CbCTB1* grown in light using WT grown in light as the control is shown as a reference. Each sample was normalized against actin control. Data represents the combination of three biological reps, each with three technical reps. Log^2 was used to model the proportional change. Error bars represent standard error.

In Δ*Cb10910* all of the genes were either repressed or not significantly different compared to the WT (Fig. 2.15). *CbCTB2*, *CbCTB5*, *CbCTB9* and *CbCTB10* were all repressed in Δ*Cb10910*. In contrast, Δ*CbCTB1* had significantly more expression in CbCTB3 than the WT. *CbCTB11* and

CbCTB12 were the only genes which were dramatically repressed compared to WT in Δ*CbCTB1*. The remaining genes in Δ*CbCTB1* did not differ significantly in expression from the WT.

Objective 4

As mentioned before, it is well known that cercosporin biosynthesis is light induced. All of the genes involved with cercosporin toxin biosynthesis have been previously shown to be upregulated in the presence of light (de Jonge et al, 2018; Chen et al, 2007). To determine if the duplicated cluster is light activated, Δ*Cb10910* and Δ*CbCTB1* from objective 3 were utilized. In WT, Δ*Cb10910,* and Δ*CbCTB1* strains, gene expression in cultures grown in light were measured using cultures grown in the dark as a control. The Δ*CbCTB1* was included as a comparison since Δ*Cb10910* is a duplication of Δ*CbCTB1*.

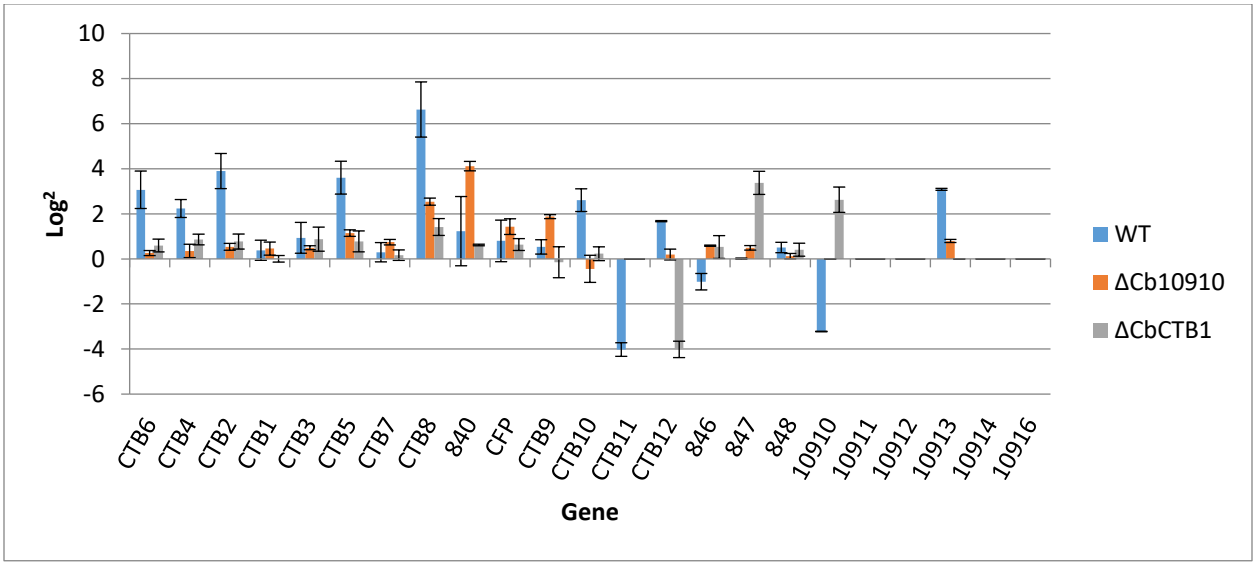

Figure 2.16. Quantitative RT-PCR analysis of Δ*Cb10910* grown in light using grown Δ*Cb10910* in dark as control. qPCR analysis of WT and Δ*CbCTB1* grown in light with dark as the control is shown as a reference Each sample was normalized against actin control. Data represents the combination of three biological reps, each with three technical reps. Log^2 was used to model the proportional change. Error bars represent standard error.

Δ*Cb10910* had lower expression than the wild type for all of the genes (Fig. 2.16). Δ*Cb10910* had comparable expression to Δ*CbCTB1* for most genes, except *Cb847* and *Cb10910*

which were higher in Δ*CbCTB1*. In contrast Δ*CbCTB1* had low levels of expression for most of the genes analyzed, except for *Cb847* and *Cb10910*.

Discussion

Many of the organisms that produce biologically toxic secondary metabolites have mechanisms to protect themselves from toxic intermediates (Daub et al, 2005). One of these mechanisms may be clustering which may facilitate strict coordination of gene expression (Keller and Hohn, 1997; Keller et al, 2005). Gene clustering may be particularly important during the biosynthesis of secondary metabolites that are chemically unstable intermediates to ensure their efficient conversion to final end products (McGary et al, 2013). Another mechanism is feedback inhibition which is the inhibition of an enzyme controlling an early stage of a series of biochemical reactions by the end product (Hubbard and Stadtman, 1966). This would mean that the process of producing a toxin would be halted before there is a buildup of toxic intermediates.

Gene regulation within the *CTB* cluster has been evidenced in *C. nicotianae* when the *CTB8* mutant failed to express genes in the *CTB* cluster (*CTB1* to *CTB7*) (Chen et al, 2007). The data presented in Fig. 2.5 shows that this holds true for *CbCTB8* in *C. beticola* as well. Furthermore, Δ*CbCTB8* also prevented expression of genes in the recently identified *CTB* genes, which further supports their contributing to cercosporin biosynthesis. The genes *Cb840*, *Cb846*, *Cb847*, and *Cb848* were all not repressed in Δ*CbCTB8*. This suggests that these genes are not involved with cercosporin biosynthesis. These results likely indicate that the Zn(II)Cys_6 zinc finger transcription factor *CTB8* mediates expression of the known *CTB* genes.

In this study we used quantitative PCR to elucidate the regulatory roles of *Cb840*, *CFP*, *CTB9*, *CTB10*, *CTB11*, *CTB12*, and *Cb846*. The absence of *CFP* suppressed the expression of all of the *CTB* genes except *CTB7* and *CTB9*. This makes sense since *CFP* encodes a major facilitator

superfamily transporter and is thought to partially provide cercosporin autoresistance through toxin export (Callahan et al, 1999). Without the autoresistance to cercosporin that *CFP* provides there could be a toxic buildup of cercosporin within the cell, so *CFP* having feedback inhibition is logical. The absence of *CbCTB9* represses several of the original cluster genes, including *CTB8*, which could prevent a buildup of potentially toxic pre-cercosporin (Fig. 2.17). In contrast, the same does not hold true for *CbCTB10*, which only significantly suppressed expression of *CTB2* and *848.* This may indicate that *CTB10* does not play as critical of a role in oxidative ring closure as *CTB9.* In the absence of *CbCTB11, CTB3* and *CTB10* were dramatically repressed. *CbCTB11* is a βig-h3 fasciclin which is predicted to dimerize cercoquinone C in conjunction with *CTB12.* It is logical that *CbCTB11* would suppress the *CTB3* since it is responsible for the step immediately prior to *CTB11*. The absence of *CbCTB12* suppressed *CTB2*, *CTB5, CTB10, 848, 10910* and *10913*. The repression of *CTB2*, *CTB5* and *CTB10* make sense since they are all steps in cercosporin biosynthesis that come after the dimerization by *CTB11/CTB12*.

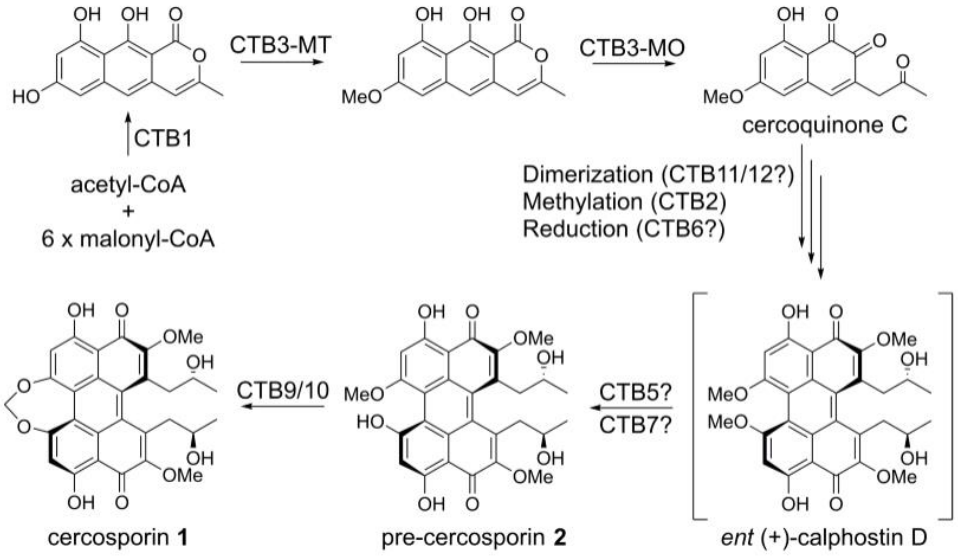

Figure 2.17. Proposed biogenesis of cercosporin. Tentative proposal for biosynthesis of cercosporin (1), incorporating newly discovered *CTB* genes. Intermediates in brackets are logically inferred, and have not been directly observed. MT = methyltransferase, MO = monooxygenase (de Jonge et al, 2018).

It currently is not known if *Cb840* or *Cb846* play any role in the biogenesis of cercosporin. *Cb840* expression was not influence by light in the WT (Fig. 2.4) and only *CbCTB2* and *CbCFP* were suppressed in its absence. *Cb840* was unaffected by the absence of *CTB8* unlike the genes that have been identified as part of the *CTB* cluster. Likewise, *Cb846* was not shown to be light activated in the WT. In the absence of *Cb846*, *CbCTB8*, *Cb840*, *CbCTB10*, *Cb848* and *Cb10913* were suppressed. However similarly to *Cb840*, *Cb846* was not affected by the absence of *CTB8*. All this information combined suggest that *Cb840* and *Cb846* are not involved with cercosporin biosynthesis.

de Jonge et al (2018) found that there was significant similarity between *CbCTB1* and *Cb10910* as well as many of the flanking genes (Fig. 2.2). They hypothesized that the *Cb10910* cluster is the result of cluster duplication. Secondary metabolite cluster duplication is a fairly rare occurrence in fungi (Medema et al, 2014). At the inception of this experiment we had two

hypotheses about *Cb10910*. One hypothesis was that *Cb10910* would be expressed similarly to *CTB1* if it is involved in cercosporin biosynthesis. The second hypothesis was that *Cb10910* would be upregulated in the absence of *CTB1* if it provides functional redundancy to *CTB1*. Functional redundancy would be advantageous for the fungus since *CTB1* is essential for cercosporin production (Newman et al, 2012; Crawford and Townsend, 2010; Choquer et al, 2005). Neither of these hypotheses were validated. *Cb10910* was suppressed in the light in the WT background unlike *CTB1* which was upregulated. This in conjunction with not having been induced by light may suggest that the suppression of *CTB* genes in Δ*Cb10910* is a pleiotropic effect rather than *Cb10910* having a direct interaction or participation in cercosporin biosynthesis. The duplicated *CTB* cluster was not highly activated in any of the knockouts or WT also suggesting that the duplicated cluster does not play a role in cercosporin biosynthesis.

Conclusions

We disrupted the *CbCTB8* gene coding for a zinc finger transcription factor and found in the resultant mutant that gene regulation occurred in all the *CTB* genes. A *CbCFP* mutant was also generated and found similar results to *CbCTB8*, a nearly complete suppression of all *CTB* genes. This complete and near complete suppression of *CTB* genes in these two mutants suggests that they are crucial to either cercosporin production or autoresistance. The remaining genes analyzed had suppression of surrounding genes some to a greater extent than others. This suggests that the suppression of genes may be a mechanism to prevent the buildup of toxic cercosporin intermediates. *Cb840* and *Cb846* mutants both suppressed selected *CTB* genes, but both genes lack of induction by light leaves uncertainty if they play a role in cercosporin biosynthesis. The *CbCTB1* duplication *Cb10910* was found to not be induced by light and was not functionally redundant to *CbCTB1* nor played an obvious role in cercosporin production. This study has shown that the

expression of *CTB* and selected surrounding genes is complex. The analysis has given further evidence that the recently identified *CTB* genes are a part of cercosporin biosynthesis. This work, combined with previous studies has shown that cercosporin production is carefully regulated by both gene regulation and feedback inhibition. The research accomplished in this study provides a better understanding of gene regulation in this important toxin.

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