IDENTIFICATION AND CHARACTERIZATION OF NOVEL CERCOSPORA BETICOLA NECROSIS-INDUCING EFFECTORS

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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

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ABSTRACT

Cercospora beticola is a hemibiotrophic fungus responsible for Cercospora leaf spot disease of sugar beet (*Beta vulgaris*). Plant pathogens such as *C. beticola* utilize "effector" molecules to aid in disease establishment. Effectors are generally small, secreted molecules that contribute to pathogen virulence. A culture filtrate infiltration study was conducted to identify potential effector molecules secreted by *C. beticola*. A variety of fungal growth conditions were pursued, one of which resulted in a necrotic phenotype when the culture filtrate was infiltrated into sugar beet leaves. The culture filtrate was fractioned using ion-exchange chromatography. Fractions were infiltrated to identify the protein responsible for necrosis. Five candidate necrosis-inducing effector proteins were identified through mass spectrometry analysis. Targeted gene disruption of these candidates and subsequent virulence assays revealed an increased virulence for Δ*05663* strains compared to the inoculated wild-type. Full characterization of this candidate effector will shed light on the *C. beticola*-sugar beet interaction.

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CHAPTER ONE: LITERATURE REVIEW

1.1. *Beta vulgaris* subsp. *vulgaris*, the host

Sugar beet, *Beta vulgaris* subsp. *vulgaris*, is an essential crop for sugar production. Aside from sugar cane, it is the only crop source for sucrose, a valuable component of the human diet (Cooke, 2008). Roughly one-third of the world's annual sugar production comes from sugar beet (Dohm et al., 2014). Sugar beet production exceeded 35 million tons in 2020 from over 1.17 million planted acres in the US (USDA-NASS, 2021). The highest production per harvested acre occurs in the Red River Valley (RRV) area of Minnesota and North Dakota, the Imperial Valley of California, southern Idaho, and northeastern Michigan (USDA-ERS, 2021). Areas of significant sugar beet production outside the US include Russia, France, and Germany (FAOSTAT, 2019).

In 1753, Carl Linnaeus first described sugar beet and assigned it to the *Beta* genus (Linné, 1764). Besides sugar beet, the *Beta* genus includes cultivar groups leaf beet, garden beet, and fodder beet (Monteiro et al., 2018), all domesticated from wild sea beet, *Beta maritima* (Biancardi et al., 2012). Cultivation of the common ancestor, *Beta vulgaris* ssp. *provulgaris*, fell into two categories: leaf beet (*B. vulgaris* ssp. *cicla*) or root beet (*B. vulgaris* ssp. *vulgaris*) (Cooke, 2008).

Origins of the sugar beet industry began in 19th century Europe. The discovery of beet sugar crystallization was critical for future sugar extraction commercialization. German scientist Andreas Marggraf is credited for comparing the crystals in sugar beet with sugar cane crystals and noticing their chemical similarities (Marggraf, 1748). However, his student Franz Carl Achard became the "father of the sugar beet industry" due to his successful selection of high sugar-yielding beet (Cooke, 2008). In 1801, Achard built the first sugar beet factory in Cunern,

Silesia, which spurred excitement in Europe for sugar beet production (Draycott, 2008). In 1811 Napoleon Bonaparte published a series of decrees, one of which established six sugar beet schools to teach manufacturing practices (Magnuson, 1918). An instructor at one of Napoleon's schools inspired two Americans, Edward Church and David Lee Child, to build the first US factory in 1839 in Northampton, MA (Magnuson, 1918). After a number of failed sugar processing factories in several US states, the first successful factory was established in 1870 in Alvarado, CA (Magnuson, 1918). Sugar beet production in the RRV area began in 1918 near Crookston, MN, although processing occurred several hundred miles away in Chaska, MN (Panella et al., 2014). After a steady production growth, the first processing plants in the RRV area were established in 1948 (Crookston) and 1954 (Moorhead), and it is now the largest region in the US for sugar beet production with a total of five sugar processing plants (Panella et al., 2014). There are now 21 major factories in nine different US states.

1.2. *Cercospora beticola*, the pathogen

Cercospora beticola is a filamentous fungus that belongs to the phylum Ascomycete, class Dothidiomycete, order Capnodiales, family Mycosphaerellaceae, and genus *Cercospora* (Goodwin et al., 2001, El Housni et al., 2021, Chupp, 1953). Species in the *Cercospora* genus are typically associated with leaf spot diseases (Groenewald et al., 2013). *Cercospora beticola* causes Cercospora leaf spot (CLS) disease on sugar beet and was first characterized in 1876 on *B. cicla* (Saccardo, 1876). Potential *C. beticola* hosts include crop and weed species such as swiss chard (McKay & Pool, 1918), spinach (Knight et al., 2019), safflower (Lartey et al., 2005), lettuce (Houessou et al., 2011), and *Acanthus mollis* (bear's breeches) (Rooney-Latham et al., 2011).

Cercospora leaf spot disease can reduce sugar beet root weight and sucrose content, contributing millions of dollars in losses per year (Skaracis et al., 2010). Foliage loss can reduce photosynthetic potential, and consequently, root sugar reserves may suffer due to vegetative regrowth (Rossi V, 2000). Root storage losses can also be a consequence of CLS because of a predisposition to decay and increased respiration (Smith & Ruppel, 1971). Some reports have implicated moderate to severe CLS outbreaks causing almost 50% loss of recoverable sucrose (Rangel et al., 2020). A 1998 CLS outbreak in the RRV area caused an estimated loss of $113 million due to yield reduction and costly fungicide use (Rangel et al., 2020).

1.2.1. *Cercospora beticola* life cycle

Cercospora beticola is known to exist only in the asexual, anamorphic stage (Weiland & Koch, 2004). The life cycle of *C. beticola* begins with overwintering pseudostromata in infested soil either on or below the soil surface (Khan et al., 2008). In the spring, melanized conidiophores are formed from conidia-producing pseudostromata (Rangel et al., 2020). Dispersal of conidia occurs through wind and rain splash (Khan et al., 2008). The disseminated spores can then land on host leaf petioles or leaves to initiate infection (Rangel et al., 2020). Germination occurs with a high humidity rate of nearly 100%, free water, and temperatures around 25°C (Khan et al., 2008). After conidia germinate, appressoria are produced that may penetrate leaf stomata (Rangel et al., 2020). Hyphae then spread intercellularly with no visible leaf symptoms (Steinkamp et al., 1979). After approximately five to 14 days, necrotic leaf spot lesions that are characteristic of the disease develop. Infected leaves and petioles exhibit dark brown to reddish/purple circular spots with a tan or ashen gray center (Windels et al., 1998). Leaf spots can coalesce and cause leaf collapse/senescence with increasing disease severity (Weiland & Koch, 2004). Pseudostromata form in these necrotic lesions and become the site for

new conidial development. Conidia are again dispersed, initiating a new infection cycle provided environmental conditions conducive to disease are present (Weiland & Koch, 2004).

1.2.2. Management of *C. beticola*

Repeated use of the same fungicide can result in pathogen resistance. Therefore, fungicide resistance management strategies involve understanding pathogen biology, knowledge of fungicide resistance development, and the mode-of-action of fungicide chemistries (Damicone, 2009). The main goal of fungicide resistance management is to slow selection for fungicide-resistant strains in a population (Bosch et al., 2014). Since the 1981 CLS epidemic in the RRV, growers have implemented various fungicide resistance management strategies, including selection of fungicide chemistries that have efficacy against *C. beticola* strains in the region, fungicide tank mixing, and alternating between different fungicide chemistries (Rangel et al., 2020, Windels et al., 1998). Unfortunately, fungicide resistance in most populations has continued to increase. The polycyclic nature of this pathogen adds to the difficulty in managing CLS outbreaks due to repeating life cycles throughout a growing season, creating more opportunity for selection pressure of beneficial mutations (Bolton et al., 2012). Benzimidazoles, demethylation inhibitors, organotin, and quinone outside inhibitors are all fungicides for which populations of *C. beticola* have shown some level of resistance (El Housni et al., 2018, Giannopolitis, 1978, Bugbee, 1995, Campbell et al., 1998, Dafang & Shuzhi, 1982, Trkulja et al., 2013, Nikou et al., 2009, Karaoglanidis et al., 2001, Secor et al., 2010, Secor et al., 2017).

The development of CLS disease prediction models helps anticipate disease severity, thereby assisting growers in understanding when to apply necessary fungicides (Windels et al., 1998). CLS epidemic severity depends on favorable environmental conditions, cultivar resistance, use and effectiveness of chemical treatments, and crop growth dynamics in and

around surrounding fields (Rossi V, 2000). Spraying before disease development and early in the season can prevent high conidial populations from developing (Rangel et al., 2020).

Reducing initial inoculum sources is also a critical disease management strategy. *Cercospora beticola* can persist in plant debris on the soil surface for the entire year (Khan et al., 2008). Crop rotation with non-host crops has been shown to lower available *C. beticola* inoculum (Weiland & Koch, 2004). Deep tillage can be beneficial because this practice buries residues of the present inoculum (Rangel et al., 2020). Additionally, planting sugar beet crop at least 100 m from a field infested with CLS the previous season can limit available inoculum due to airborne conidia traveling via rain splash or on farm machinery (Windels et al., 1998).

Another component of CLS management is the use of disease-resistant sugar beet varieties. In 1932, sugar beet was crossed with wild sea beet in Italy to increase the genetic basis of sugar beet (Munerati, 1932). Since that time, sea beet has been an important source of genetic resistance to CLS. For example, many *B. vulgaris* ssp. *maritima* accessions have exhibited strong resistance to CLS and have been used to introgress new disease resistance genes into sugar beet (Luterbacher et al., 2004). However, some CLS-resistant varieties may have a yield penalty in the absence of significant disease pressure (Rossi et al., 2000, Weiland & Koch, 2004). Fortunately, a recent study shows promise for reducing the sugar yield penalty observed in CLS-resistant sugar beet varieties (Vogel et al., 2018).

1.3. Plant-pathogen interactions

1.3.1. Gene-for-gene interactions

In Flor's gene-for-gene hypothesis, a specific host resistance (R) protein recognizes an avirulence (Avr) molecule from the pathogen, which then confers resistance to the disease (Flor, 1956). Fungal effectors, which may inadvertently act as avirulence determinants, are generally

described as cysteine-rich (≥2 cysteine residues), small in size (≤300 amino acids), and secreted during host infection (Stergiopoulos & de Wit, 2009). Although disparate in size, molecule type, delivery mechanism, and classification of fungi that secrete them, effectors share a common role in helping to establish disease. Recognition of effectors typically occurs through plant R proteins containing nucleotide-binding leucine-rich repeat domains. For example, in rice and the infecting pathogen *Magnaporthe oryzae*, resistance protein RGA5 represses RGA4 in the absence of recognizable avirulence effectors. However, recognition of *M. oryzae* effector Avr-Pita by RGA5 relieves RGA4 repression, resulting in programmed cell death (PCD) and resistance to the pathogen (Cesari et al., 2014). PCD, a hallmark of R gene-mediated resistance, is a host immune response that can restrict pathogen ingression to the neighboring cells when specific dominant host genes recognize pathogen effectors (Coll et al., 2011). Interactions between pathogen *Cladosporium fulvum* and host tomato also demonstrate a gene-for-gene interaction. Effectors Avr2, Avr4, Avr4E, and Avr9 of *C. fulvum* are recognized by tomato cognate resistance genes Cf-2, Cf-4, Cf-4E, and Cf-9, respectively (de Wit et al., 1997, Joosten & de Wit, 1999, Stergiopoulos & de Wit, 2009). The interaction between these R genes and effectors has been a valuable model for studying R-mediated defense responses and R gene evolution (Parniske & Jones, 1999, Kruijt et al., 2004, Rivas & Thomas, 2005).

1.3.2. Inverse gene-for-gene interactions

In contrast to Flor's gene-for-gene hypothesis, some necrotrophic fungi are involved in an 'inverse gene-for-gene' (IVGG) interaction. In an IVGG interaction, susceptibility, rather than resistance, is the outcome of pathogen recognition by the host (Richards et al., 2017). Some necrotrophic and hemibiotrophic pathogens take advantage of the host immunity system in the IVGG model. Such pathogens can tolerate the host PCD response and use dead host tissue as a

nutrient source (Faris & Friesen, 2020). For example, *Stagonosporum nodorum* uses multiple effectors to initiate PCD by triggering host susceptibility (S) genes, which are necessary for disease to occur (Friesen et al., 2008, Friesen et al., 2007). Consequently, absence of either host S gene or the pathogen effector will result in disease resistance (Friesen et al., 2008).

1.4. *Cercospora beticola* effector molecules

Plant pathogens secrete effector molecules to establish disease. While various "omics" techniques have recently expanded our knowledge base of effectors in well-studied model interactions, characterization of the *C. beticola* effector repertoire is in its infancy. So far, two secondary metabolites (SMs) and three proteinaceous effectors are described in *C. beticola*.

1.4.1. *Cercospora beticola* secondary metabolite effectors

Secondary metabolites are low-molecular-weight compounds that are not essential for fungal growth and development (Keller et al., 2005). However, they can be involved in other processes, including virulence and toxicity (Rangel et al., 2021, Pusztahelyi et al., 2015). In *C. beticola*, two well-known SMs are cercosporin and beticolin (Daub & Ehrenshaft, 2000). Cercosporin is a light-activated toxin synthesized by the cercosporin toxin biosynthesis cluster consisting of at least twelve genes (de Jonge et al., 2018). In the presence of oxygen, cercosporin can produce reactive oxygen species, which is toxic to the host cell (Daub et al., 2005). Targeted gene replacement studies show cercosporin can be a critical virulence factor for *Cercospora* species (Weiland et al., 2010).

Beticolin molecules are non-host-specific SMs with antibacterial and phytotoxic properties (Goudet et al., 1999). Beticolins can cause a range of effects, including loss of solutes *in planta* (Schlösser, 1971), inhibition of ATP-dependent H+ transport (Macri et al., 1983, Blein et al., 1988), membrane depolarization (Gapillout et al., 1996), or *in vitro* O_2 scavenging activity

(Rustérucci et al., 1996). Like cercosporin, the phenotypic response beticolin can cause in plants only occurs in the presence of light (Schlösser, 1962). However, because the biosynthetic pathway is currently unknown, it is not yet possible to determine to what extent beticolin contributes to *C. beticola* virulence.

1.4.2. *Cercospora beticola* protein effectors

Cercospora beticola necrosis-inducing protein 1 (CbNip1) is crucial for disease development in sugar beet (Ebert et al., 2021). Because the necrotic response caused by CbNip1 was enhanced in the absence of light, it was postulated this effector complements light-dependent cercosporin activity (Ebert et al., 2021). Additionally, *CbNip1* resides in a genomic area that underwent a selective sweep. Selective sweeps are distinct genomic signatures indicative of a positively selected mutation spread that occurred in a population (Spanner et al., 2021). Based on its location within a selective sweep, there was likely selection pressure to maintain CbNip1 in the population (Ebert et al., 2021). Lastly, it is posited CbNip1 is non-host specific because of the general cytotoxicity of the protein (Ebert et al., 2021).

Cladosporium fulvum Avr4 is an effector protein that has been shown to bind fungal chitin, which is thought to prevent a chitin-mediated resistance response in the host tomato (van den Burg et al., 2003). A PCR-based approach identified homologs of Avr4 in several *Cercospora* species, including CbAvr4 in *C. beticola* (Mesarich et al., 2016). The Cf-4 immune receptor cannot recognize CbAvr4, and consequently, Cf-4-dependent PCD is not triggered (Stergiopoulos et al., 2010). Results from the Mesarich et al. (2016) study suggest CbAvr4 lacks a conserved proline residue required to trigger Cf-4-dependent hypersensitive response (Mesarich et al., 2016). More experimental data is needed to confirm whether CbAvr is a core effector with a chitin-binding function in *C. beticola*.

Secretion of *Verticillium dahliae* effector VdAve1 triggers immunity in tomato plants encoding the Ve1 immune receptor (Song et al., 2018). A homolog of VdAve1, designated CbAve1, was identified in *C. beticola* and determined to be a *C. beticola* virulence factor (de Jonge et al., 2012, Boshoven et al., 2015). Immune receptor Ve1 can recognize CbAve1, suggesting Ve1-mediated immune signaling is an evolutionarily conserved pathway across plant species (Song et al., 2018). Studies involving transgenic expression of cell surface immune receptor genes suggest immune receptors such as Ve1 could be a valuable resource in disease resistance (Rodriguez-Moreno et al., 2017).

1.5. Identification of necrosis-inducing effectors using culture filtrate infiltration

Culture filtrates are the *in vitro* cultivation of microbial organisms in liquid resulting in an accumulation of a complex set of proteins in the media (Sonnenberg & Belisle, 1997). Infiltration of culture filtrates can provide a visual representation of interactions occurring between host and pathogen within intercellular leaf space. A needleless syringe containing culture filtrate is typically used to apply pressure and introduce the liquid (Chincinska, 2021). Post-culture infiltration, researchers can analyze host phenotypic changes such as necrotic tissue damage that result from culture filtrate activity. Subsequently, strategies to identify the pathogen effector responsible for necrosis may include protein separation through chromatography, mass spectrometry analysis of peptides, and subsequent analyses of gathered protein data to determine which molecule in the cultured media is responsible for the phenotypic change.

Culture filtrate studies in 1987 initiated the eventual discovery of ToxA in *Pyrenophora tritici-repentis* (Tomas & Bockus, 1987). In this study, resistant and susceptible wheat cultivars were infiltrated with culture filtrates from *P. tritici-repentis* isolates. These wheat cultivars were also inoculated with spores from the same isolates. Resistant wheat lines were asymptomatic,

whereas susceptible lines showed necrotic symptoms (Tomas & Bockus, 1987). The small, secreted protein from this initial study was eventually purified and identified as ToxA (Zhang et al., 1997, Tuori et al., 1995, Ballance et al., 1996). Necrosis-inducing proteins ZtNip1 and ZtNip2 were also identified using liquid chromatography and mass spectrometry analysis of fractioned *Zymoseptoria tritici* culture filtrates (M'Barek et al., 2015). ZtNip1 is homologous to *C. fulvum* effector Ecp2, which can elicit tomato cell death (Laugé et al., 1998, M'Barek et al., 2015). ZtNip2 has an MD-2-related lipid-recognition domain with potential roles in innate immunity (M'Barek et al., 2015). In the M'Barek et al. (2015) study, upregulation of multiple necrosis-inducing proteins may be responsible for triggering host defense responses, switching the pathogen from a biotrophic to a necrotrophic lifestyle (Friesen & Faris, 2021).

1.6. Molecular editing tool CRISPR/Cas9

Clustered regularly interspaced short palindromic repeats (CRISPR)- associated RNA-guided Cas9 nuclease (CRISPR/Cas9) has recently become a revolutionary gene-editing tool. CRISPR regions were first discovered in *Escherichia coli* in 1987 (Ishino et al., 1987). After observing CRISPR sequences in other bacteria and archaea species, researchers concluded spacer sequences within CRISPRs were derived from plasmid and viral origins, suggesting the CRISPR/Cas9 system was implemented for defense purposes in bacteria (Doudna & Charpentier, 2014). In the bacterial CRISPR/Cas9 system, a single RNA molecule uses a linker sequence to join trans-activating CRISPR RNA (tracrRNA) and target CRISPR RNA (crRNA) molecules. Scientists manipulated this editing tool in *Streptococcus pyogenes*, where a small-guide RNA (sgRNA) was created by fusing crRNA and tracrRNA. The DNA of interest is targeted by sgRNA and subsequently binds to the protospacer adjacent motif sequence (Foster et al., 2018, Jinek et al., 2012). The result is a CRISPR/Cas9 complex that creates double-strand breaks

(DSBs) in the target DNA sequence (Komor et al., 2017, Shi et al., 2017). DSBs are repaired through homology-directed repair, which can introduce specific desired mutations or sequences using donor DNA templates, often carrying dominant selectable markers, disrupting the coding sequence of the gene of interest (Sander & Joung, 2014).

The first successful editing using CRISPR/Cas9 in a fungal species was in *Trichoderma reesei* (Liu et al., 2015b). By 2019, the CRISPR/Cas9 editing system had been implemented in over 40 different filamentous fungi species (Schuster & Kahmann, 2019). Recently, a Cas9 purified nuclear localization sequence (Cas9-NLS) and *in vitro* synthesized sgRNA complex called ribonucleoprotein (RNP)-CRISPR/Cas9 was developed (Foster et al., 2018), which was adapted for *C. beticola* to assess virulence of mutants with disrupted candidate necrosis-inducing effector genes (this thesis). The RNP-CRISPR/Cas9 method uses PCR-amplified donor DNA, eliminating the need for labor-intensive DNA cloning, and has few off-target sites, demonstrating high efficiency.

1.7. Future perspectives

Molecular advances will help manage CLS disease and understand the interaction between sugar beet and *C. beticola*. Recent access to reference quality genomes of sugar beet and *C. beticola* are important for future breeding efforts (Dohm et al., 2014, de Jonge et al., 2018, Vaghefi et al., 2017). Effector molecules may also be used to assist in breeding practices. Previous research has demonstrated identification of effector molecules can help identify S genes in respective hosts. Breeders can use the identified S genes for genetic plant protection as a potentially more durable form of host resistance (Engelhardt et al., 2018). CRISPR/Cas9 can be used for the modification of S genes. For instance, mildew resistance locus O (MLO) is a plant S gene that has been modified with CRISPR/Cas9 in tomato, wheat, and grapevine (Wang et al.,

2014, Malnoy et al., 2016, Nekrasov et al., 2017). A transgene-free tomato plant created using CRISPR/Cas9 to edit the *SlMlo1* locus resulted in resistance to powdery mildew disease (Nekrasov et al., 2017). Because S genes like MLO can be conserved throughout different species, S gene modification through CRISPR/Cas9 may offer opportunities for resistance to multiple pathogens. CRISPR/Cas9 has also corrected defective R genes. For example, the substitution of a G > A using this base editing technology was achieved in rice, consequently recovering the biological function of the R gene *pi-d2* (Ren et al., 2018). The vast applications of CRISPR/Cas9 will help decipher plant-pathogen interactions and improve breeding for more pathogen-resistant crops.

CHAPTER TWO: IDENTIFICATION AND CHARACTERIZATION OF NOVEL CERCOSPORA BETICOLA NECROSIS-INDUCING EFFECTORS

2.1. Abstract

Cercospora beticola is a hemibiotrophic fungus responsible for Cercospora leaf spot disease of sugar beet (*Beta vulgaris*). Plant pathogens such as *C. beticola* utilize "effector" molecules to aid in disease establishment. Effectors are generally characterized as small, secreted molecules that contribute to pathogen virulence. A culture filtrate infiltration study was conducted to identify potential effector molecules secreted by *C. beticola*. A variety of fungal growth conditions were pursued, one of which resulted in a necrotic phenotype when the culture filtrate was infiltrated into sugar beet leaves. The culture filtrate was fractioned using ion-exchange chromatography, and fractions were then infiltrated into sugar beet leaves to identify the protein responsible for necrosis. Three culture filtrate fractions were sent for mass spectrometry analysis, identifying five candidate necrosis-inducing effector proteins. Targeted gene disruption of these candidates and subsequent virulence assays displayed an increase in virulence for Δ*05663* strains demonstrated by higher levels of disease severity on inoculated sugar beet when compared to the wild-type strain. Full characterization of this candidate effector will shed light on the *C. beticola*-sugar beet interaction.

2.2. Introduction

Cercospora beticola is a hemibiotrophic pathogen with an initial biotrophic stage that occurs before an unknown trigger causes the pathogen to switch to a necrotrophic lifestyle (Rangel et al., 2020). In the symptomless biotrophic stage, appressoria penetrate sugar beet leaf cells, facilitating intercellular hyphal growth with no visible *in planta* symptoms, while *C. beticola* gains nutrients from living plant tissues. (Weiland & Koch, 2004). In the necrotic stage,

host cell death occurs through the secretion of fungal degradative enzymes, phytotoxins, and effectors (Ebert et al., 2021, de Jonge et al., 2012, Mesarich et al., 2016, Rangel et al., 2020, Koeck et al., 2011). Effectors are small, diverse molecules secreted by pathogens to aid disease establishment (Stergiopoulos & de Wit, 2009). For example, secretion of secondary metabolites (SMs) cercosporin and beticolin likely benefit *C. beticola* in nutrient acquisition and disease proliferation due to their phytotoxic properties (Daub & Ehrenshaft, 2000, Goudet et al., 1999). Because it is a hemibiotroph, it is conceivable *C. beticola* requires different effector repertoires for biotrophic and necrotrophic stages. For example, the effector repertoire of hemibiotroph *Phytophthora capsici* consists of apoplastic and intracellular effectors (Jupe et al., 2013). Apparent transcriptional shifts suggest secretion of *P. capsici* effectors during biotrophy suppress host defense responses, and effector genes expressed late in infection are utilized for cell death and modulation of host metabolism (Jupe et al., 2013). Expression pattern profiling in other hemibiotrophic pathogens has led to candidate effector identification and provided evidence for when the switch from biotrophy to necrotrophy occurs (Haddadi et al., 2016).

The proteome of *C. beticola* has previously been assessed for putative effectors. These analyses identified 1,087 secreted *C. beticola* proteins that lack a transmembrane domain, a feature of protein effectors (Franceschetti et al., 2017, Rangel et al., 2020). Of these proteins, 333 were predicted to be effector molecules due to their small size (≤300 amino acids) and rich cysteine content (≥2 cysteine residues) (Rangel et al., 2020). Currently, only two characterized SMs and three proteinaceous effectors have been described in *C. beticola* (Ebert et al., 2021, Boshoven et al., 2015, Mesarich et al., 2016, de Jonge et al., 2012). The first characterized necrosis-inducing effector protein in *C. beticola* was CbNip1, identified via culture filtrate infiltration studies (Ebert et al., 2021). The identification of CbNip1 demonstrated necrosis-

inducing *C. beticola* effectors are released into culture media (Ebert et al., 2021). Other hemibiotrophic pathogen effectors have been identified in culture filtrate studies. For example, analysis of *Z. tritici* culture filtrates identified two necrosis-inducing proteins ZtNip1, and ZtNip2 (M'Barek et al., 2015).

The main objective of this study was to identify *C. beticola* necrosis-inducing effectors secreted *in vitro* through culture filtrate infiltration experiments. To prevent the necrosis-inducing properties of CbNip1 from interfering with the results of this study, we used *C. beticola* Δ*CbNip1*, a strain unable to produce the CbNip1 protein. We fractioned *C. beticola* Δ*CbNip1* culture filtrate harvested from specific culturing regimes and assayed fractions for necrosis properties by infiltration into sugar beet leaves. Three fractions were selected for mass spectrometry analysis, followed by protein identification using the sequenced *C. beticola* genome (de Jonge et al., 2018). Putative effectors were annotated using predictive protein ontologies, and an RNP-CRISPR/Cas9 gene-editing system was established to disrupt candidate effector genes. No virulence penalties were observed for three candidate effector gene mutants when inoculated on sugar beet. However, we observed increased virulence upon inoculation for one candidate effector gene mutant compared to the *C. beticola* wild-type strain. Identifying effectors and other pathogen defense-related properties can help piece together the nuances of host plant resistance and susceptibility (Horbach et al., 2011).

2.3. Materials and methods

2.3.1. Culture filtrate preparation and infiltration

To begin each growth condition, a 5 mm fungal plug was taken from a *C. beticola* Δ*CbNip1* (Ebert et al., 2021) strain growing on 1X potato dextrose agar (Difco Laboratories, NJ, USA) amended with 150 µM hygromycin B (MilliporeSigma, MA, USA) and used to inoculate

250 mL conical flasks containing 100 mL of Fries media (Liu & Friesen, 2012). Cultures were grown and harvested under selected conditions (shaking at 150 rpm at room temperature, grown in 24 hr darkness or 24 hr light, sampled at time points 7, 14, or 21 days after medium inoculation). In all cases, harvest of the liquid culture began with a centrifugation step at 4,000 x *g* for 10 minutes to obtain a mycelia-free supernatant which was subsequently filter-sterilized with a 0.45 µM syringe filter (Celltreat, MA, USA).

Sugar beet cultivar C093 were grown for infiltration experiments in a greenhouse chamber at 21°C with 10 hr:14 hr light:dark conditions and 70% humidity. Using a 1 mL needless syringe (Henke-Sass Wolf, Tuttlingen, Germany), 30–50 µl of sterile culture filtrate was infiltrated into the youngest fully developed leaves of seven-week-old sugar beet plants. To serve as a control, 50 µL of Fries medium was similarily infiltrated. Infiltration experiments were repeated at least three times with multiple individually produced culture filtrates. Infiltrated plants were kept in a growth chamber in a 16 hr:8 hr light:dark cycle and were monitored daily for necrosis.

2.3.2. Culture filtrate fractionation and infiltration

Culture growth conditions were identified that allowed for reproducible necrosis after infiltration of crude culture filtrate (see Results). Crude culture filtrates for downstream analyses were purified as previously described (Liu et al., 2009). Briefly, 100 mL of 7-day-old *C. beticola* Δ*CbNip1* grown in Fries medium was centrifuged at 4,000 x *g* for 10 minutes to obtain a mycelia-free supernatant, which was subsequently filter-sterilized using a 0.45 µM PVDF membrane (MilliporeSigma, MA, USA). The sterile culture filtrate was dialyzed overnight against water using dialysis tubing with a 3.5 kDa molecular weight cut-off (Fisher Scientific, NJ, USA). After 16 hrs, the dialyzed culture filtrate sample was loaded onto a HiPrep SPXL

16/10 cation exchange column on the AKTA Prime Plus fast protein liquid chromatography system (GE Healthcare, NJ, USA). After loading the sample, the column was initially washed with 15 mL of 20 mM sodium acetate buffer (pH 5.0). One mL fractions were collected during gradient elution of 0–300 mM NaCl plus 20 mM sodium acetate (pH 5.0) at a 1 mL/min flow rate over 20 minutes. Individual fractions were grouped as follows: fractions 4-6 (group 1), fractions 7-9 (group 2), fractions 10-12 (group 3) and fractions 13-15 (group 4). These four groups were infiltrated as described above. Fractionation and infiltration experiments were repeated at least three times. The infiltrated plants were maintained as described previously.

2.3.3. Preparation of necrosis-inducing protein fraction for MS/MS analysis

Individual fractions 4 to 15 were loaded onto a precast 4-20% Mini-PROTEAN TGX gel (Bio-Rad, CA, USA) that included the Benchmark Protein Ladder (10-220 kDa) (Invitrogen, MA, USA) to visualize protein band profiles. Fractions from groups 1 and 2 caused necrosis when infiltrated into sugar beet leaves (see Results). Therefore, individual fractions 5, 6, and 7 were sent to the Center for Mass Spectrometry and Proteomics at the University of Minnesota for tandem mass spectrometry (MS/MS) analysis. Identification of proteins was achieved using Multidimensional Protein Identification Technology (Washburn et al., 2001) using the *C. beticola* proteome of wild-type strain 09-40 (de Jonge et al., 2018). The MS/MS data analysis revealed six candidate proteins: CB0940_05663, CB0940_06172, CB0940_06327, CB0940_02286, CB0940_03645, and CB0940_08676.

2.3.4. Sequence analysis

Putative functionality of candidate effectors was initially achieved via NCBI BLASTp analyses using the non-redundant protein sequence database. Protein domain families were classified using the Pfam database (v. 34.0; http://pfam.xfam.org/). SignalP was used to

decipher if traditional standard secretory peptides were present using the Eukarya organism group and long output format (v. 5.0; http://www.cbs.dtu.dk/services/SignalP). EffectorP was used to predict the likelihood of the candidates being fungal effectors and was used to distinguish between apoplastic and cytoplasmic effectors (v. 3.0; http://effectorp.csiro.au//). Software DiANNA was used to predict if cysteines were involved in the formation of disulfide bonds (v. 1.1; http://clavius.bc.edu/~clotelab/DiANNA/).

2.3.5. Candidate protein deletion mutants

In order to assess whether the candidate effectors play a role in virulence, three individual mutants in each candidate gene were developed using either split-marker (Bolton et al., 2016) or RNP-CRISPR/Cas9 methodologies (Foster et al., 2018). *Cercospora beticola* Δ*06172* mutants were generated with the split-marker method using primers listed in Table 1. *Cercospora beticola* Δ*05663*, Δ*06327*, and Δ*08676* mutants were generated following the RNP-CRISPR/Cas9 protocol using primers listed in Table 1. For the generation of RNP-CRISPR/Cas9 components, potential off-target sites were assessed using EnsemblFungi (https://fungi.ensembl.org/). The E-CRISP website (http://www.e-crisp.org) using *Magnaporthe oryzae* (closest relation to *C. beticola*) identified a protospacer adjacent motif (PAM) site. Single guide RNA selection for complexing to Cas9-NLS was achieved using the New England Biolabs online tool (https://sgrna.neb.com/#!/sgrna). Single guide RNA (sgRNA) synthesis was conducted according to the EnGen sgRNA synthesis kit protocol (New England Biolabs, MA, USA) and purified using the RNA Clean and Concentrator-25 kit (Zymo Research, CA, USA) before complexing to Cas9-NLS (New England Biolabs, MA, USA) in a ten-minute incubation at room temperature. Single guide RNAs were always freshly synthesized and purified on the day of transformation. Donor DNA templates were designed using 40 bp flanks of either side of

the PAM cut site with complementary hygromycin b phosphotransferase sequences added to the end of the forward and reverse primers.

Table 1. Primers used in this study

Primer	Name	Sequence (5′ => 3′)
MDB- 258	Split- marker HY	GGATGCCTCCGCTCGAAGTA
MDB- 259	Split- marker YG	CGTTGCAAGACCTGCCTGAA
MDB- 277	Split-marker M13F	GACGTTGTAAAACGACGGCCAGTG
MDB- 278	Split-marker M13R	CACAGGAAACAGCTATGACCATGA
MDB-1145	HY-R2	GGCAGGTAGATGACGACCAT
MDB-2722	06172_1F	GTAGCAACGTCCAGGAAAGG
MDB-2723	06172_2R	CACTGGCCGTCGTTTTACAACGTCAGAACGCAGTGCAAGAAGGT
MDB-2724	06172_3F	TCATGGTCATAGCTGTTTCCTGTGTGCGTGCCGTACTAGAGTTG
MDB-2725	06172_4R	GCGACTTTGTTGTTGGAGGT
MDB-2721	06172_5'1F	GAGTTCTCGATCGTGGCATT
MDB-2810	sgRNA 06327	TTCTAATACGACTCACTATAGCTGGGCACAAAGACATTCGGTTTTAGAGCTAGA
MDB-2811	Donor-F_06327	ATCGCCTCCGCCAATGATGGCACCCAGAACAATCCCGCGAGACGTTGTAAAACGACGGCCAGTG
MDB-2812	Donor-R_06327	ATCTGGTACCAAAATGGGACGCCGCTGGGCACAAAGACATCACAGGAAACAGCTATGACCATGA
MDB-2813	Whole- F_06327	CTCGAATTCACGACACGAGA
MDB-2814	Whole- R_06327	TCTGCCAGGAGCTCAAAAGT
MDB-2701	06327_5'1F	GGAGAGGGACAAAGACATGC
MDB-2815	sgRNA 03645	TTCTAATACGACTCACTATAGGGGGGAGGGGAGGTGCGGAGTTTTAGAGCTAGA
MDB-2816	Donor-F_03645	CCAACGCCAACCTGGCAAATCATCCTCTCCTCCACCCTCCGACGTTGTAAAACGACGGCCAGTG
MDB-2817	Donor-R_03645	ATGATGGAGTAGTTTGAAGAAGAGGGGGGAGGGGAGGTGCCACAGGAAACAGCTATGACCATGA
MDB-2839	Whole- F_03645	ACACCCTCTCCCTCCTCCT
MDB-2840	Whole- R_03645	CCCGTTTTTCAGACCGATT
MDB-2706	03645_5'1F	GACTTCGTCTTTTCCGGTCA

Table 1. Primers used in this study (continued)

Primer	Name	Sequence (5′ => 3′)
MDB-2841	sgRNA-08676	TTCTAATACGACTCACTATAGACGACTACGTGGATTATGAGT TTTAGAGCTAGA
MDB-2842	Donor-F_08676	TGGACCAAGCCCTTCGGATCTTTGACGACTACGTGGATTAG ACGTTGTAAAACGACGGCCAGTG
MDB-2843	Donor-R_08676	CCTCGAAGTATCCTGGGCTTGCTGAGGAGTGACACCATCAC ACAGGAAACAGCTATGACCATGA
MDB-2844	Whole-F_08676	GTTCAAGCCCAACTTCTCCA
MDB-2845	Whole-R_08676	ACGTTCGTAGCACCGTATCC
MDB-2711	08676_5'1F	AGCTGTATAACCCGCGACTG
MDB-2872	sgRNA-05663	TTCTAATACGACTCACTATAGAGCCAGACGTGCGGCCGCAG TTTTAGAGCTAGA
MDB-2873	Donor-F_05663	TCAATATGCTTACTCCAGCTCTTACTTTCGCCGTCCTTGCGA CGTTGTAAAACGACGGCCAGTG
MDB-2874	Donor-R_05663	AGTCGTACTGGCATACTGAGCTTGAGCCAGACGTGCGGCCC ACAGGAAACAGCTATGACCATGA
MDB-2875	Whole-F_05663	CGCTCCTACAAAGGATCGAG
MDB-2876	Whole-R_05663	CAGCCGAGACTGTGAACGTA
MDB-2716	05663_5'1F	GCACAGTGTCGACTTTTGGA
MDB-2877	sgRNA-06172	TTCTAATACGACTCACTATAGATGAAGTCGGCAAAGGCTCG TTTTAGAGCTAGA
MDB-2878	Donor-F-_06172	CCTGCGGACTACTTCTTCAGCAGCGATGCTAACGCCCGAGG ACGTTGTAAAACGACGGCCAGTG
MDB-2879	Donor-R-_06172	TTCACCGGACCCCAGGCACCCCAGATGAAGTCGGCAAAGGC ACAGGAAACAGCTATGACCATGA
MDB-2880	Whole_F_06172	CCCTTGCGGCTACTATCATT
MDB-2881	Whole_R_06172	GCAGAACGCATTGTGGTG
MDB-2721	06172_5'1F	GAGTTCTCGATCGTGGCATT

2.3.6. Fungal transformation and introduction of DNA templates

The previously described polyethylene glycol (PEG)-mediated transformation (Bolton et al., 2016) generated split-marker PCR or RNP-CRISPR/Cas9 mutants. For the CRISPR/Cas9 transformation, RNP complexes (Cas9-NLS complexed to sgRNA) and donor DNA templates were introduced simultaneously before the step where 50% PEG is added, and the mixture is incubated for 25 min. Donor DNA and Cas9-NLS were mixed at a 1:1 molar ratio with the respective sgRNA. After about seven days, cultures from the transformant plates appeared and were transferred to PDA amended with 150 µM hygromycin B. After another five to seven days, a 5 mm plug of the site-directed transformant culture (Δ*05663*, Δ*06172*, Δ*06327*, or Δ*08676*) was excised from the PDA-hygromycin plate and sliced into small pieces, which were used to inoculate 100 mL Fries medium in a 250 mL conical flask. Cultures were grown at 25 °C for seven days, shaking at 150 rpm. Cultures were filtered through Miracloth (MilliporeSigma, MA, USA), flash-frozen in liquid nitrogen, then ground using a mortar and pestle. Genomic DNA was extracted using a modified version of the DNeasy Plant Mini Kit protocol (Qiagen, MD, USA). Three individual mutants for Δ*05663*, Δ*06172*, Δ*06327*, and Δ*08676* were confirmed with PCR using the primers listed in Table 1. Sanger sequencing (Eurofins Genomics LLC, KY, USA) confirmed a single hygromycin integration and absence of gene amplicons (Figures 1 and 2).

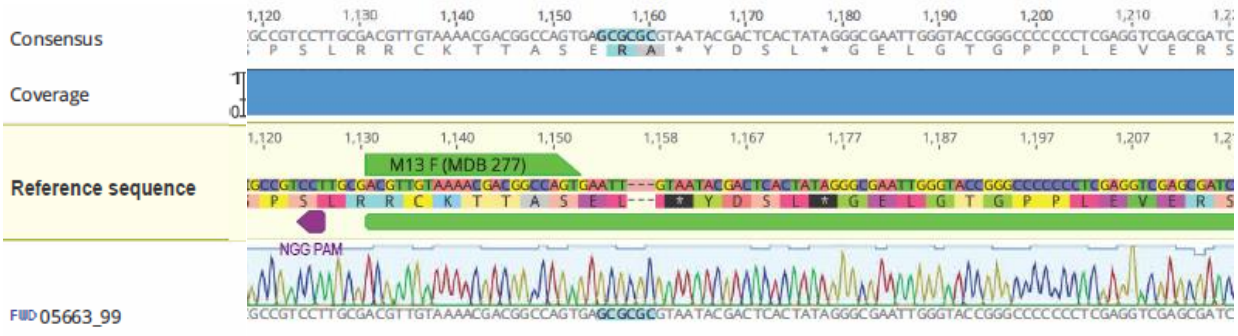


Figure 1. Confirmation of site-directed integration of the hygromycin resistance gene inserted via RNP-CRISPR/Cas9. Alignment of an individual Δ*05663* strain to the reference sequence using the 5' flanking region of gene target *CB0940_05663*. The protospacer adjacent motif (PAM) is indicated by the purple triangle. Insertion of hygromycin occurred 4 bp downstream of PAM. Beginning of the hygromycin sequence indicated by forward M13 primer (MDB 277; Table 1).

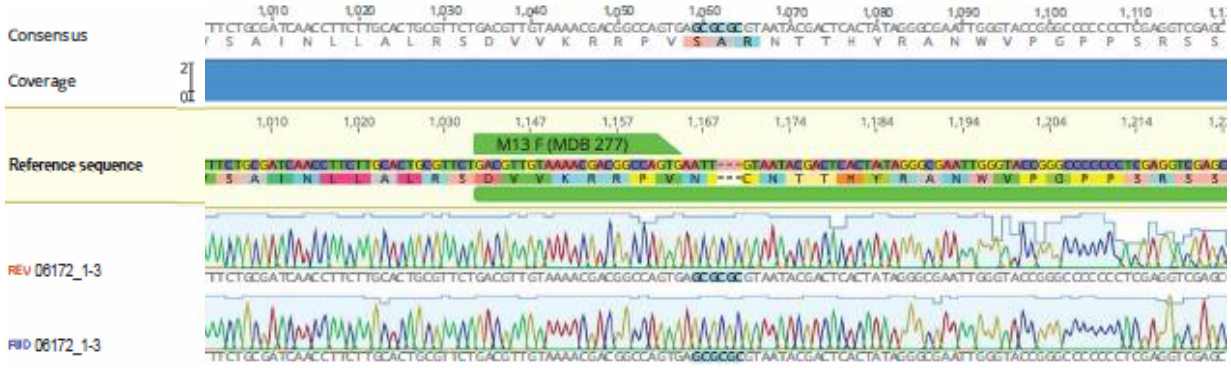


Figure 2. Confirmation of site-directed integration of the hygromycin resistance gene inserted via split-marker PCR. Alignment of an individual Δ*06172* strain to the reference sequence built using the 5' flanking region of the gene target *CB0940_06172*. Beginning of the hygromycin sequence indicated by forward M13 primer (MDB 277; Table 1).

2.3.7. Inoculation assay

Spore formation of *C. beticola* wild-type 09-40 and three individual mutants for Δ*05663*, Δ*06172*, Δ*06327*, or Δ*08676* were produced on CV8 agar plates following a previously described protocol (Secor & Rivera, 2012). Spores were harvested and adjusted to a concentration of 10^5 spores/mL. Sugar beet plants were placed in a spray booth (DeVries Manufacturing, Hollandale, MN) and sprayed with spore suspensions of approximately 22 mLs at 3.2 km and 0.2 MPA on the leaves of 7-week-old sugar beet plants (variety C093), grown as previously described, for disease assays. Inoculated plants were kept in a humidity chamber of

approximately 90% humidity for seven days. Plants were then transferred to a greenhouse with a 12 hr:12 hr light:dark cycle and closely monitored for visible symptom formation. Three leaves from each of three plants were harvested two weeks post-inoculation for each of three replications. Disease severity for Δ*06172*, Δ*06327*, and Δ*08676* was assessed by calculating the mean lesion area of scanned leaves using American Phytopathological Society Assess 2.0 imaging software (Lamari, 2008). For Δ*05663*, disease severity was assessed by counting *C. beticola* lesions and assigning each leaf to a category using the Kleinwanzlebener Saatzucht (KWS) rating scale (Kleinwanzlebener Saatzucht, 1970). On the KWS scale, category 1 has fewer than five spots and 0.1% severity, and category 10 is over 200 spots and 50% disease severity (Kleinwanzlebener Saatzucht, 1970).

2.4. Results

2.4.1. *Cercospora beticola* culture filtrate Δ*CbNip1* can induce necrosis in sugar beet plants

Cercospora beticola secretes necrosis-inducing effector CbNip1 during *in vitro* growth (Ebert et al., 2021). To uncover additional necrosis-inducing effectors, *C. beticola* Δ*CbNip1* was cultured in Fries medium under different conditions (e.g., shaking at 150 rpm at room temperature, sampling time points at 7, 14, and 21 days after medium inoculation, and growing in 24 hr light or 24 hr dark conditions) in attempts to identify *in vitro* conditions at which necrosis-inducing effector proteins were produced. Presence of necrosis-inducing activity was evaluated for all culture conditions by infiltrating culture filtrate into sugar beet leaves (Figure 3A). Infiltration assays revealed culture filtrate of *C. beticola* Δ*CbNip1* grown in Fries medium for seven days, shaking at 150 rpm at room temperature, and in 24 hr darkness, caused repeatable necrosis of the host tissue after seven days (Figure 4). Ion-exchange chromatography was performed on the active Δ*CbNip1* culture filtrate to single out the protein responsible for the

necrotic phenotype (Figure 5B). The four fraction groups were screened for necrosis-inducing activity by infiltration into sugar beet leaves (Figure 6). Additionally, fractions were individually analyzed on an SDS-PAGE gel (Figure 5A). Infiltration of fraction groups 1 and 2 reproducibly displayed the strongest necrotic phenotype and contained individual fractions that displayed protein band profiles that differed from the other fractions (Figure 5A). Due to these results, fractions 5, 6, and 7 were selected for protein identification using tandem mass spectrometry (MS/MS) analysis.

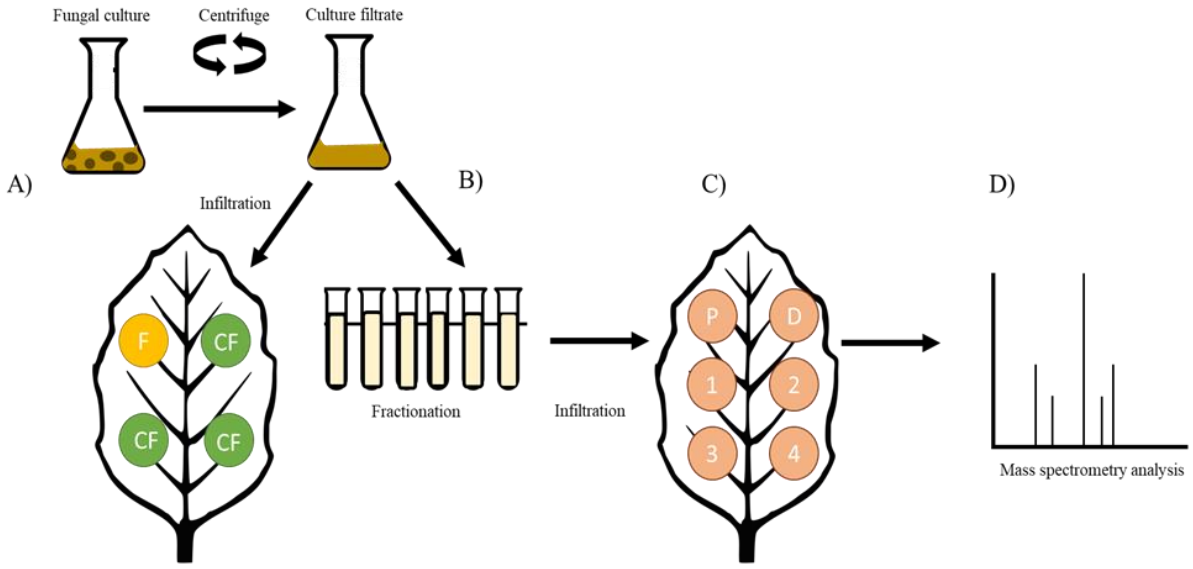


Figure 3. Pipeline for necrosis-inducing effector identification. A) A seven-day-old *Cercospora beticola* Δ*CbNip1* was grown in Fries medium and spun down to retrieve the supernatant and remove fungal mycelia. Infiltration of the culture filtrate (CF) into seven-week-old sugar beet leaves resulted in a necrotic phenotype after seven days. Infiltration of Fries media control (F) did not produce a necrotic phenotype. B) Culture Δ*CbNip1* was fractioned with ion-exchange chromatography. C) Groups of fractions (1-4), pre-dialysis (PD), and dialyzed (D) samples were infiltrated into sugar beet leaves, and necrosis-inducing activity was assessed. D) Fractions with the strongest necrosis-inducing activity were sent for mass spectrometry analysis. All infiltration experiments were repeated three times using different sugar beet plants.

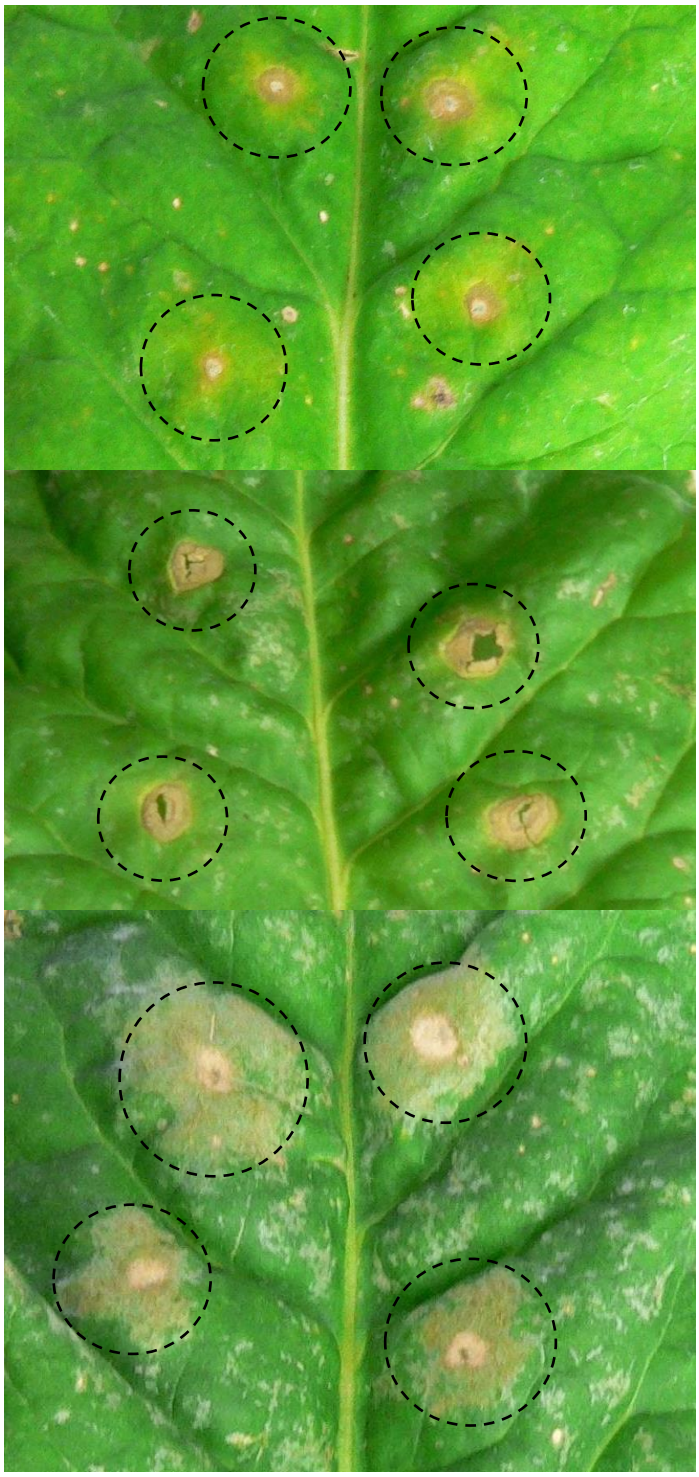
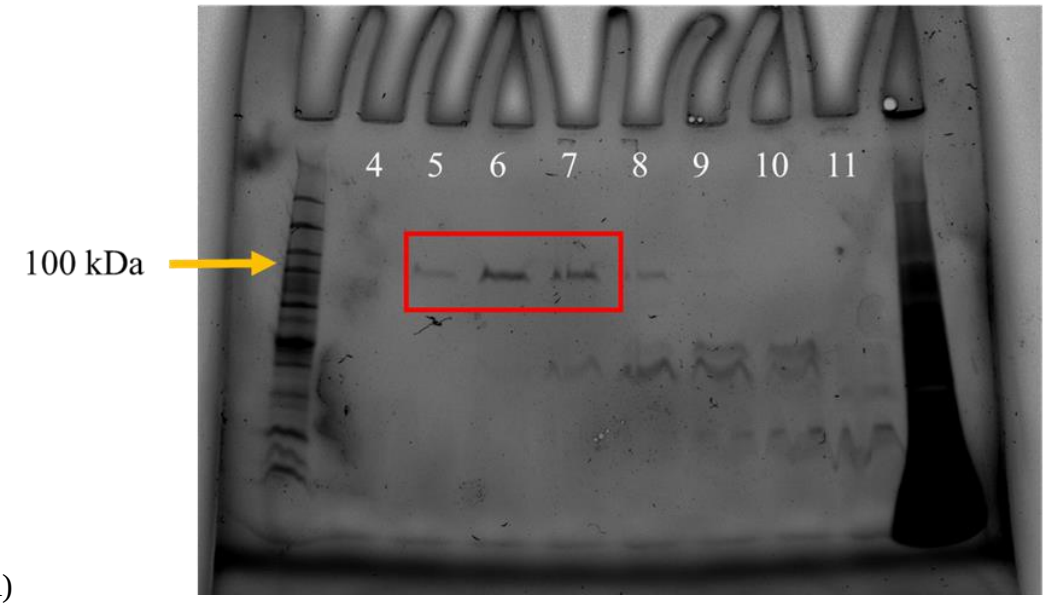


Figure 4. Necrotic activity of *C. beticola* Δ*CbNip1*. Chlorosis/necrosis development seven days after infiltration of Δ*CbNip1* into sugar beet leaves (adaxial side shown). Fries media infiltration served as a control, and the infiltrated area remained unchanged (not shown). Plants were kept in a 16 hr:8 hr light:dark cycle. All infiltration experiments were repeated at least three times using different sugar beet plants.

A)



B)

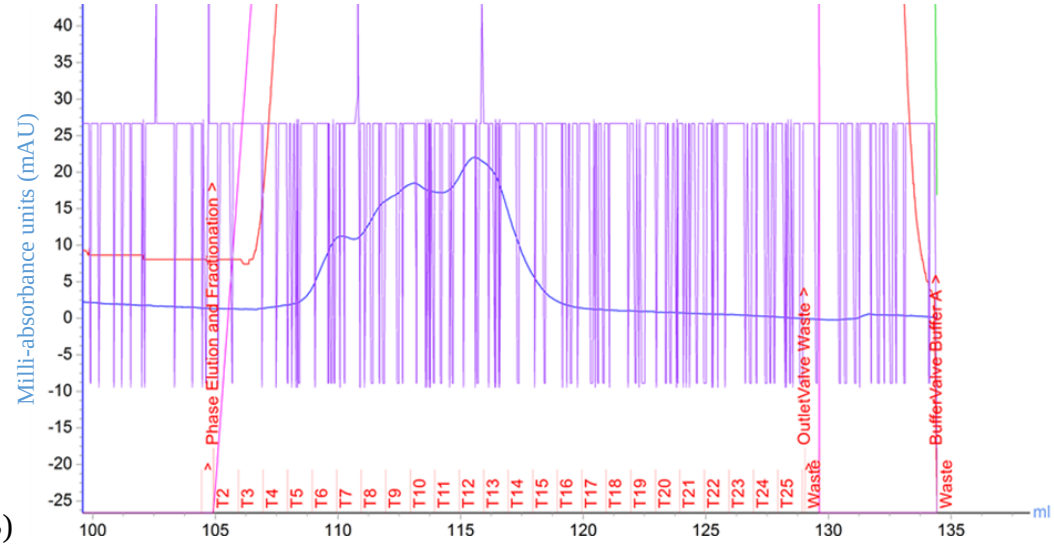


Figure 5. A) SDS-PAGE gel displaying protein content of fractions 4-11. Individual fractions 5, 6, and 7 sent were for MS/MS analysis, indicated by the red box. B) Chromatogram obtained during gradient elution of Δ*CbNip1* culture filtrate sample at 28 dpi. The blue line represents the UV curve at which a peak was detected in fraction tubes 4–15.

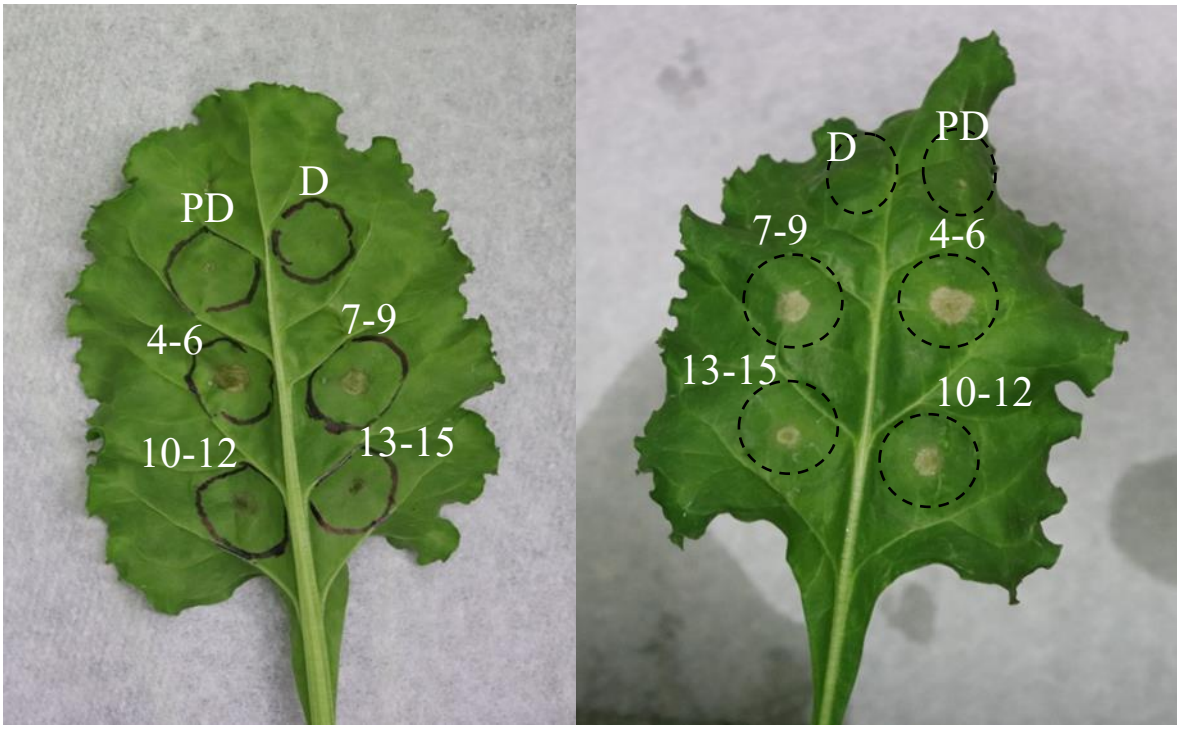


Figure 6. Infiltration of fractioned Δ*CbNip1* culture filtrate. Culture filtrate Δ*CbNip1* was fractionated using ion-exchange chromatography. Individual fractions were combined into four groups and assayed by infiltration for necrosis-inducing activity after 24 hrs. Infiltrated sugar beet leaves were kept in growth chambers in a 16 hr:8 hr light:dark cycle. Pre-dialysis (PD) and dialyzed (D) samples were also infiltrated. All infiltration experiments were repeated at least three times using different sugar beet plants.

2.4.2. Characterization of putative effector proteins

Based on MS/MS data analysis and subsequent protein identity searches in the genome of *C. beticola* strain 09-40 (de Jonge et al., 2018), six candidate proteins were identified: CB0940_05663, CB0940_06172, CB0940_06327, CB0940_02286, CB0940_03645, and CB0940_08676. Among the candidate proteins, CB0940_02286 lacked a signal peptide and was therefore excluded from further analysis. Of the five candidate proteins, four candidates displayed high cysteine content (≥2 cysteine residues) (Table 2). Analysis of the five candidates with effector prediction software EffectorP v. 3.0 identified CB0940_06327 and CB0940_05663 as putative apoplastic effectors (Table 2).

Table 2. Summary of the top five candidate effectors and associated effector characteristics

Protein number	Pfam domain	EffectorP 3.0	Protein size (aa)	Molecular weight (kDa)	Cysteine residue	Disulfide bridges
CB0940_05663	Glycosyl hydrolases family 16	Apoplastic effector	257	27.27	0	0
CB0940_06172	Glycosyl hydrolase family 18	No prediction	277	29.67	9	4
CB0940_06327	F5/8 type C domain	Apoplastic effector	558	60.58	2	1
CB0940_03645	Glycoside-hydrolase family GH114	No prediction	276	30.61	6	3
CB0940_08676	Pectate lyase	No prediction	837	90.23	10	5

2.4.3. *Cercospora beticola* Δ*05663* increased virulence upon inoculation on sugar beet cultivar C093

To gain insight into whether each candidate is required for *C. beticola* virulence, we generated gene disruption mutants for CB0940_05663, CB0940_06327, CB0940_06172, and CB0940_08676. Sugar beet plants were inoculated with a wild-type *C. beticola* strain and three individual Δ*05663*, Δ*06327*, Δ*06172*, or Δ*08676* strains to assess visible *in planta* symptoms. Disease progression and visual symptom development of sugar beet plants inoculated with wild-type *C. beticola*, and three individual Δ*06172*, Δ*06327*, or Δ*08676* strains was variable but did not differ from the wild-type (Figures 7, 8, and 9). At nine days post-inoculation, sugar beet plants inoculated with three individual Δ*05663* strains showed higher levels of disease severity in comparison to the wild-type *C. beticola* strain (Figure 10).

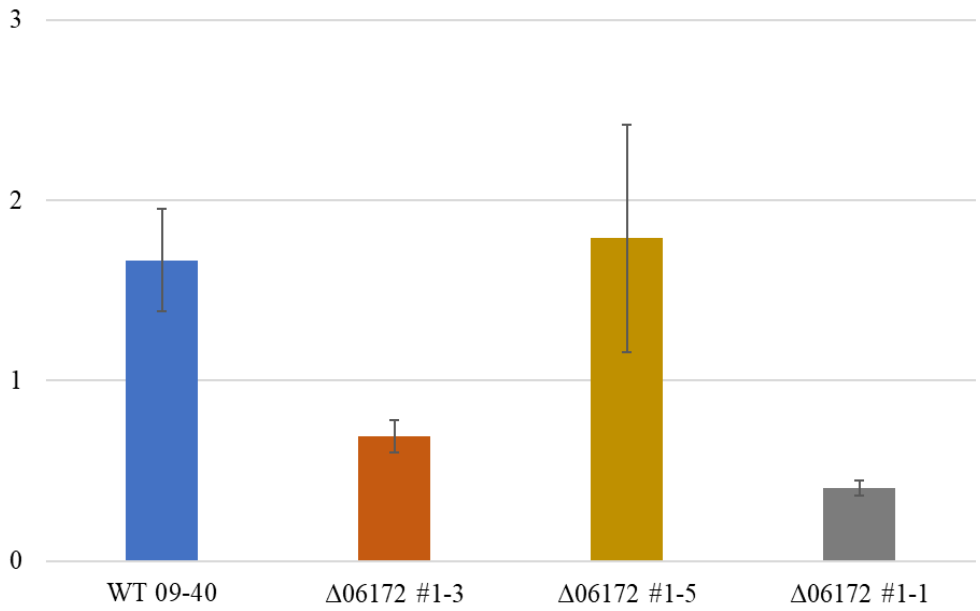


Figure 7. Disease development during infection of *Cercospora beticola* 09-40 wild-type strain and three individual Δ*06172* mutants on sugar beet. Disease severity was assessed by calculating the mean percentage of diseased area (displayed on the y-axis) using APS Assess imaging software (Lamari, 2008). Sugar beet plants inoculated with *C. beticola* strains were harvested at 15 days post-inoculation. Error bars represent the standard error of the three biological replicates.

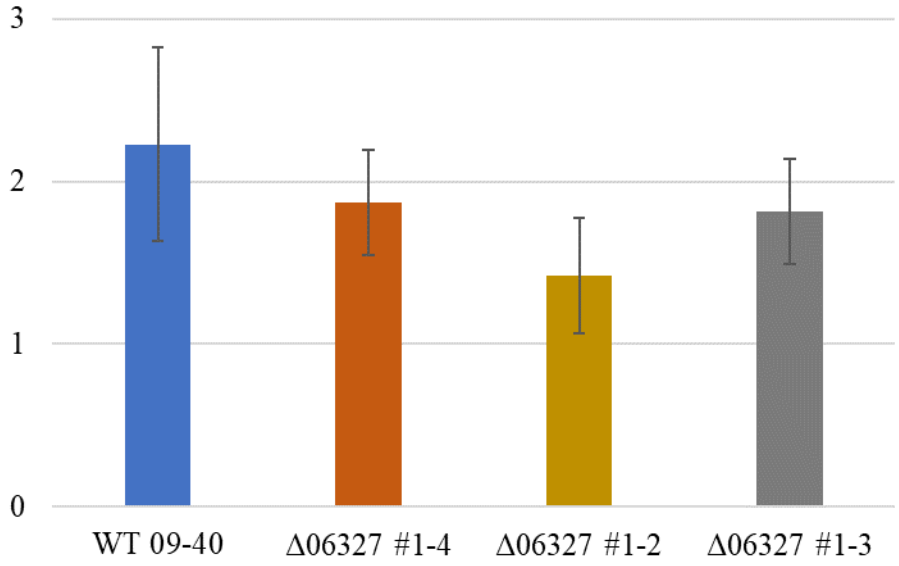


Figure 8. Disease development during infection of *Cercospora beticola* 09-40 wild-type strain and three individual Δ*06327* mutants on sugar beet. Disease severity was assessed by calculating the mean percentage of diseased area (displayed on the y-axis) using APS Assess imaging software (Lamari, 2008). Sugar beet plants inoculated with *C. beticola* strains were harvested at 17 days post-inoculation. Error bars represent the standard error of the three biological replicates.

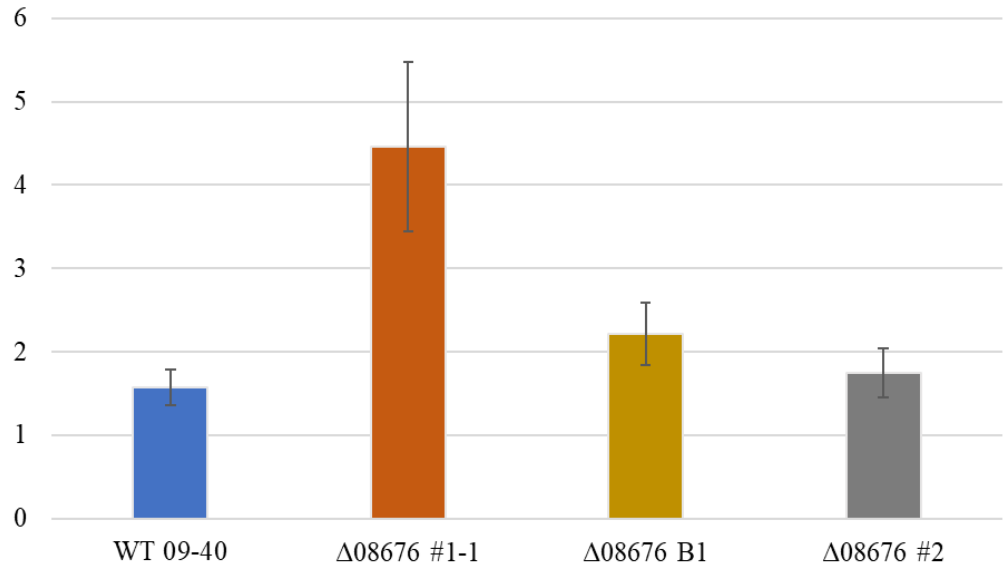


Figure 9. Disease development during infection of *Cercospora beticola* 09-40 wild-type strain and three individual Δ*08676* mutants on sugar beet. Disease severity was assessed by calculating the mean percentage of diseased area (displayed on the y-axis) using APS Assess imaging software (Lamari, 2008). Sugar beet plants inoculated with *C. beticola* strains were harvested at 21 days post-inoculation. Error bars represent the standard error of the three biological replicates.

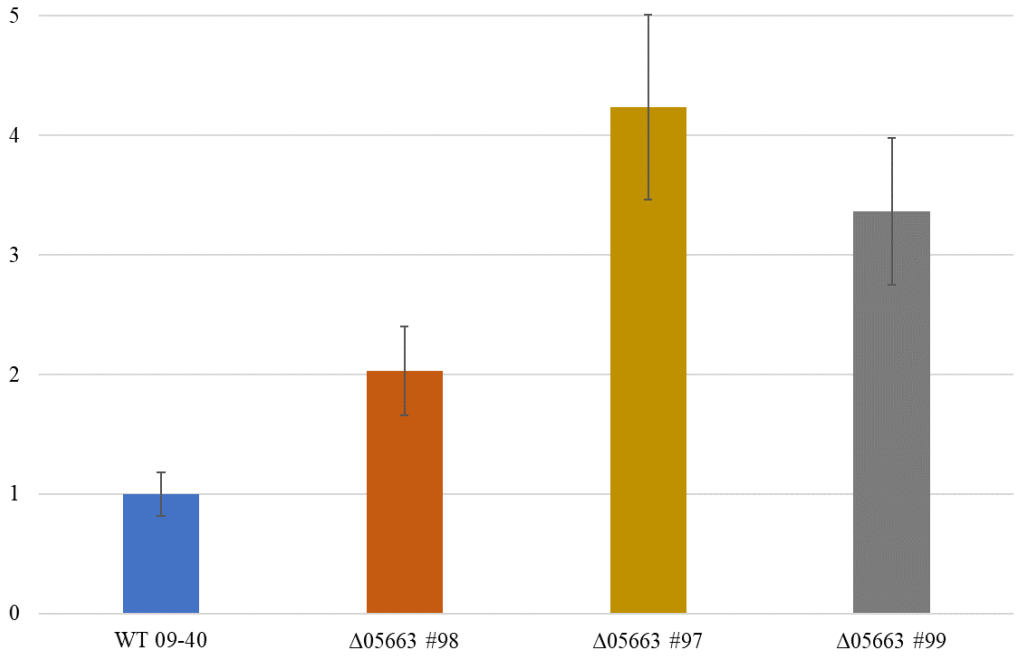


Figure 10. Disease development during infection of *Cercospora beticola* 09-40 wild-type strain and three individual Δ*05663* mutants on sugar beet. Disease severity was assessed using the 1-10 KWS rating scale to calculate the mean disease severity (displayed on the y-axis) (Kleinwanzlebener Saatzucht, 1970). Sugar beet plants inoculated with *C. beticola* strains were harvested at nine days post-inoculation. Error bars represent the standard error of the three biological replicates.

2.5. Discussion

Despite the impact of effectors on sugar beet-*C. beticola* interactions, most of the *C. beticola* effector repertoire has yet to be characterized. Identification and characterization of effectors can improve upon our knowledge of existing molecular interactions and help influence future disease management strategies. In this study, we report the identification of five putative necrosis-inducing effector proteins. By searching for *in vitro* parameters that induce *C. beticola* effector secretion, we established growth conditions at which culture filtrates of *C. beticola* Δ*CbNip1* possess necrotic activity seven days post-infiltration into sugar beet leaves. We used *C. beticola* Δ*CbNip1* because it cannot produce the mature CbNip1 protein, and we did not want the necrosis-inducing properties of CbNip1 to interfere with the results of this study. Fractionation of the culture filtrate followed by mass spectrometry analysis of three necrosis-inducing fractions identified six candidate effectors. Of the six proteins, CB0940_02286 was eliminated for further investigation because it lacked a traditional secretion signal. Candidates CB0940_06172 and CB0940_03645 are smaller than 300 amino acids and are cysteine-rich (Table 2). Three of the putative effectors are cysteine-rich or small in size, but not both. However, not all characterized effectors fit into effector classification regimes. To illustrate, *Melampsora lini* effector AvrM has only one cysteine residue (Catanzariti et al., 2006), and *Ustilago maydis* effector Rsp3 has 869 amino acids (Ma et al., 2018).

CB0940_05663 is classified as a glycosyl hydrolase family 16 (GH16) protein. In *Cronartium ribicola*, the causal agent of white pine blister rust, RNA-seq technology revealed GH16 proteins are highly up-regulated in early infection stages and are proposed to be cell wall modifiers for more effective fungal penetration (Liu et al., 2015a). GH16 proteins in pathogenic fungi *Cochliobolus lunata* and *Bipolaris maydis* are also plant cell wall modifiers (Gao et al.,

2014). Among five virulent *Pyrenophora teres* f. *teres* isolates, GH16 proteins represented a large proportion of the secretome (Ismail & Able, 2016), two of which were closely related to Crh1 from *Magnaporthe grisea*, which function in appressorial formation (Franck et al., 2013).

CB0940_06172 is classified as a glycosyl hydrolase family 18 (GH18) chitinase. GH18 proteins can function in tissue degradation, developmental regulation, and immune/pathogenicity defense (Huang et al., 2012). GH18 endochitinase ScCts1p is required for chitin degradation in cell separation of *Saccharomyces cerevisiae* (Kuranda & Robbins, 1991). Other GH18 chitinases are involved in sporulation (Dünkler et al., 2008) and hyphal growth (Dünkler et al., 2005). CB0940_03627 is characterized as an F5/8 type C domain protein, a coagulation factor among the motifs at least twice as common in secretomes of phytopathogenic fungi compared to non-phytopathogenic fungi (Soanes et al., 2008).

CB0940_03645 is a glycoside hydrolase family 114 (GH114) protein. In *Phytophthora cinnamomic*, one of the genes involved in plant cell wall degradation is a GH114 protein (Hardham & Blackman, 2018). In *Aspergillus fumigatus*, Ega3 belongs to the GH114 family and is involved in biofilm formation (Bamford et al., 2019). Protein CB0940_08676 is in the pectate lyase superfamily. Pectate lyases are generally among the first cell wall degrading enzymes secreted by pathogens to disassemble the host cell wall matrix (Lionetti et al., 2012). In *Erysiphe cichoracearum*, pectate lyase PMR6 is required for susceptibility in *Arabidopsis* and is necessary to promote fungal growth or activate host defenses (Vogel et al., 2002). In initial *Botrytis cinerea* infection stages, two pectate lyases were highly up-regulated, suggesting their role in pathogen cell wall degradation (Zhang et al., 2020).

To assess whether the candidates played a role in virulence, we inoculated three individual mutants of Δ*05663*, Δ*06172*, Δ*06327*, and Δ*08676* and a *C. beticola* wild-type strain.

We found disease severity of sugar beet plants inoculated with Δ*06172*, Δ*06327*, and Δ*08676* was variable but did not differ from the wild-type strain. Although we could not attribute a direct role in virulence under our assay conditions, these putative effectors may be functionally redundant with other *C. beticola* effectors. In functional redundancy, unrelated effectors could converge on a single host target, or several effectors with similar functions could target different steps in plant immunity (Win et al., 2012). Consequently, the deletion of a functionally redundant effector may not result in an obvious phenotype. For example, single-effector gene knockout mutants in *Ustilago maydis* did not affect virulence; however, double knockout strains were non-pathogenic (Müller et al., 2008).

In the gene-for-gene theory, host recognition of the pathogen effector triggers inducible defenses such as programmed cell death (PCD) (Flor, 1942, Heath, 2000). Unexpectedly, three individual Δ*05663* mutants showed higher levels of disease severity nine days post-inoculation compared to the *C. beticola* wild-type strain (Figure 10). This observed phenotype may suggest that *CB0940_05663* is an avirulence (Avr) effector gene recognized by a resistance (R) gene in sugar beet operating in a gene-for-gene interaction. Using this hypothesis, disruption of *CB0940_05663* consequently removes effector recognition and the subsequent host response, thus explaining why we observed higher disease severity on Δ*05663* sugar beet plants. In gene-for-gene interactions between *M. oryzae* and host rice, a gain-of-virulence was observed in isolates with disrupted effector genes *AVR-Pita* or *AVR-Pia* on cultivars containing corresponding R genes *Pita* and *Pia* (Kang et al., 2001, Miki et al., 2009).

If CB0940_05663 does operate in a gene-for-gene interaction, an open question is why this effector is maintained as an avirulence factor? Selection pressure for recognized fungal effectors to avoid detection is high, and there are many documented cases of adaptive loss of

effector genes or mutations to overcome host resistance (Sánchez-Vallet et al., 2018). One theory could be that the effector gene has multiple intrinsic functions that aid the pathogen in disease establishment, despite being a recognized effector. SnTox1 effector of *P. nodorum* has two known functions: induction of cell death in *Snn1* wheat lines and chitin-binding to prevent degradation from wheat chitinases (Liu et al., 2012, Liu et al., 2016). Another reason for maintaining this Avr effector could be drawn from the gene-for-gene interaction between *M. lini* and flax. High protein sequence variation exists in *M. lini* AvrL567 genes and corresponding flax R proteins, indicating a coevolutionary arms race that positively selects for Avr variants (Dodds et al., 2006). A fully avirulent *M. lini* phenotype can be converted to a partial virulence phenotype with a single amino acid change (Wang et al., 2007). Due to the number of AvrL567 variants, researchers speculated losing the Avr genes has a fitness penalty for *M. lini*, and variant Avr forms have a positive fitness value (Dodds et al., 2007). It could be possible that CB0940_05663 in *C. beticola* is a variant of the Avr gene, and there is partial resistance occurring. Subsequent selection in the *C. beticola*-sugar beet pathosystem could result in further sequence changes that eventually eliminate CB0940_05663 recognition by the R protein. Otherwise, it could be advantageous for *C. beticola* to keep the Avr gene, similar to the proposed theory for AvrL567 variants (Dodds et al., 2007).

In contrast to CB0940_05663 being an Avr variant with partial resistance, it is also possible there has not been enough selection pressure in this interaction to cause adaptive changes. In this theory, *C. beticola* can tolerate the host defense responses from CB0940_05663 recognition. In hemibiotroph *Colletotrichum graminicola*, the presence of host defense responses does not eliminate the ability to cause disease (Vargas et al., 2012). In *Z. tritici*-wheat pathosystem, recognition of avirulence effector Avr3D1 triggers a host defense response, yet

pathogen infection and reproduction on infected wheat are uninhibited (Meile et al., 2018). AvrD31 recognition renders partial quantitative resistance in wheat lines containing the corresponding R protein, then AvrD31 becomes downregulated in the necrotic stage (Meile et al., 2018). Perhaps as the infection progresses in hemibiotrophs, mounting pressure from host defense mechanisms causes the pathogen to switch to a more efficient necrotic stage (Kabbage et al., 2015).

This study developed an RNP-CRISPR/Cas9 protocol using the previously described methods adapted for PEG-mediated transformation of *C. beticola* (Foster et al., 2018). Using this method, we achieved successful gene disruption of three candidate effectors. To our knowledge, this is the first successfully implemented CRISPR/Cas9 strategy for creation of *C. beticola* mutants. Gene *CB0940_06172* was disrupted through split-marker PCR as previously described (Bolton et al., 2016). We were unsuccessful in disrupting *CB0940_03645* using either editing method. It is unclear why attempts to disrupt *CB0940_03645* failed. It is possible the ideal guide RNA/donor template combination was not attained, or *in vivo* editing of *CB0940_03645* in *C. beticola* is lethal.

Further research goals include infiltration of purified CB0940_05663 protein into sugar beet and other species, such as *N. benthamiana*. Observation of a PCD response post-infiltration will help demonstrate a gene-for-gene interaction is occurring. Studies showing expression of *CB0940_05663* during different infection time points could provide insight into whether this effector is up-regulated at earlier time points and down-regulated in the necrotic stage, similar to *Z. tritici* AvrD31 (Meile et al., 2018). We can also perform pull-down assays to evaluate if CB0940_05663 directly interacts with plant-derived proteins. Characterization of a direct interaction of host targets with CB0940_05663 has important implications. Identifying a

corresponding host gene is helpful for future breeding efforts to manage CLS disease. In wheat, R genes have been widely used in breeding for crop protection. Recent research has developed polygenic resistance gene stacks and introduced them into wheat lines for highly durable resistance against *Puccinia graminis* f. sp. *tritici* (Luo et al., 2021). Resistance gene stacks are one way to combat the rapid evolution of pathogen virulence. In addition to corresponding R genes, breeders can also use susceptibility (S) genes in breeding efforts. For example, ToxA in *P. tritici-repentis* has a corresponding S gene *Tsn1*. Wheat breeders can use ToxA to screen wheat germplasm and eliminate susceptible lines. Removal of *Tsn1* has improved tan spot disease (caused by *P. tritici-repentis*) with no corresponding yield impact and a general enhancement to tan spot resistance (Oliver et al., 2014, See et al., 2018). Future work to characterize the other candidate proteins in this thesis and evaluate their potential as virulence factors are required. Expression *in planta* for each candidate effector would be an interesting addition to this study, as it may demonstrate correlation between gene expression and symptom development. Finally, transient gene expression of candidates into *N. benthamiana* via *Agrobacterium*-mediated infiltration followed by apoplast extraction from infiltrated leaves could be used directly for host phenotyping.

2.6. Conclusion

We used culture filtrate infiltration assays to show that *C. beticola* Δ*CbNip1* secretes effectors *in vitro* with the ability to cause necrosis in sugar beet leaves within seven days. After culture filtrate fractionation and subsequent mass spectrometry analysis, we characterized five putative effectors using predictive protein ontology software. A plasmid-free RNP-CRISPR/Cas9 method was developed for *C. beticola* to disrupt three of the five candidate effector genes. We showed that efficient gene editing could be achieved by introducing purified Cas9 premixed to

sgRNA to form ribonucleoproteins (RNPs). This editing method can be quick and low-cost when used with PCR-generated donor DNA containing complementary sequences to a resistance marker. After inoculation of four gene candidate mutants, inoculated Δ*05663* strains displayed increased virulence on sugar beet compared to the *C. beticola* wild-type strain, suggesting candidate effector CB0940_05663 may act as an avirulence factor of *C. beticola*. Future studies will be directed at demonstrating there is a true gene-for-gene interaction occurring and identifying the corresponding host protein.

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