EFFECTS OF SYNONYMOUS AND NONSYNONYMOUS *CYP51* MUTATIONS ON DMI RESISTANCE IN *CERCOSPORA BETICOLA*

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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 the most devastating foliar pathogen of sugar beet. This pathogen is primarily managed by the application of fungicides, including demethylation inhibitors (DMIs). Given the broad use of DMIs in managing Cercospora leaf spot, resistance has developed. Recently, five haplotypes of *CbCyp51* have been correlated with DMI resistance. To improve our understanding of these haplotypes, this study has evaluated *CbCyp51* expression across haplotypes with and without DMI exposure using RT-qPCR. Significant differences were found between haplotypes in control and difenoconazole groups, but the broader implications were unclear. This study also produced mutant *C. beticola* strains with replaced *CbCyp51* haplotypes. DMI sensitivity was assessed, revealing dramatic changes in difenoconazole resistance. Most mutants exhibited elevated tetraconazole resistance, which was largely shown to be associated with the transformation process rather than haplotype exchange. Further studies involving the different haplotypes and mutants could improve our understanding of these DMI resistance associated mutations.

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1. CHAPTER ONE: LITERATURE REVIEW

1.1. The sugar beet, *Beta vulgaris* subsp *vulgaris*

Sugar beet, *Beta vulgaris* subsp. *vulgaris,* is one of two major crops grown for sucrose production across the globe (Misra and Shrivastava 2022). While the other major crop, sugar cane, can only be grown in tropical climates, sugar beet is readily grown in temperate climates. This makes sucrose production more accessible to countries with colder climates, such as Russia, France, Germany, and the United States. Sugar beet presently accounts for about 20% of the world's sucrose supply (Misra and Shrivastava 2022; ISO 2024), and the United States is one of the leading producers of the crop. In 2023, the US produced over 35 million tons of sugar beet, according to the USDA (USDA-NASS 2024). The top growing area in the US is the Red River Valley (RRV) region of Minnesota and North Dakota, which contributed 18.7 million tons of sugar beet in 2023 (W.P.R. 2024). Outside the US, the top producers of sugar beet include Russia, France, and Germany (FAOSTAT 2022).

The scientific classification of sugar beet is *Beta vulgaris* ssp. *vulgaris* (Monteiro et al. 2018). The species name is shared with several other cultivars, including other 'root beets' (*B. vulgaris* ssp. *vulgaris*) garden beet and fodder beet, as well as the leaf beet (*B. vulgaris* ssp. *cicla*), swiss chard (Lange et al. 1999; Biancardi et al. 2012). The current varieties of domesticated *B. vulgaris* share a common wild ancestor *B. vulgaris* ssp. *maritima*, the wild sea beet, which was first domesticated about 2500 years ago (Lange et al. 1999; Monteiro et al. 2018). Despite the long history of beet cultivation, sugar beet is one of the most recently developed crops, tracing its origins to the mid-18th century (Zeven and Harten 1979).

The discovery that sugar could be extracted from beet was made by German chemist Andreas S. Marggraf of Berlin University in 1747 (Marggraf 1748; Magnuson 1918). This work

was initially inconsequential until his student, Franz Karl Achard, improved upon Andreas' methods, developing a process for large scale sugar extraction from beets in 1799 (Magnuson 1918). With support from Frederick William III, King of Prussia, this led to the building of the world's first sugar beet factory in Cunern, Silesia, with a processing capacity of 4,400 sugar beets per day (Magnuson 1918). Reports of this success spread across Europe, and the process received great interest from Napolean Bonaparte, as France was reliant on the West Indies for sugar supply (Magnuson 1918). Napoleon ordered a rapid industrialization of sugar beet production and sugar extraction (Magnuson 1918). By 1813, there were 334 sugar beet processing factories in operation in France, which produced an output of 7,700,000 pounds of refined sugar that year (Magnuson 1918). The production of beet sugar subsequently spread to many other countries (Magnuson 1918). In the United States, numerous attempts at producing a successful sugar beet industry were made after 1830, but it was not until 1870 that the first successful beet sugar factory was built in Alvarado, California (Magnuson 1918). The first sugar beet crop in the RRV was grown in 1918, and the first processing plant was built near East Grand Forks in 1926 (Panella et al. 2014). Factories have since been built near Wahpeton, Crookston, Hillsboro, Moorhead, and Drayton, helping the RRV become the largest sugar beet production area in the United States (Panella et al. 2014).

1.2. *Cercospora beticola*, sugar beet pathogen

Cercospora beticola is a filamentous, hemibiotrophic fungus in the phylum Ascomycota, class Dothidiomycete, order Capnodiales, family Mycosphaerellaceae, and genus *Cercospora* (El Housni et al. 2021). It is the causal agent of Cercospora leaf spot disease (CLS) on sugar beet, one of its primary hosts. *C. beticola* was first described in 1876 on *B. vulgaris* ssp. *cicla* by Saccardo (1876) in Italy, and has subsequently spread around the world, now infecting about one third of

sugar beet production area (Rangel et al. 2020; Tan et al. 2023). Potential hosts of *C. beticola* include other members of the *Beta* genus such as swiss chard and spinach (Knight et al. 2019), the crops lettuce (Houessou et al. 2011), safflower (Lartey et al. 2005), and various weeds such as *Chenopodium* and *Amaranthus* species (Rooney-Latham et al. 2011).

1.2.1. Life cycle of *Cercospora beticola*

Cercospora beticola begins its life cycle from pseudostromata overwintered in dead sugar beet leaf tissue (Khan et al. 2008). Pseudostromata consist of hyphae sporting asexual conidia, interwoven with the host tissue (Khan et al. 2008). Pseudostromata generate conidia in the spring, which are discharged through wind, rain, or mechanical force (Khan et al. 2008; Tan et al. 2023). Conidia germinate primarily on the abaxial side of sugar beet leaves under the condition of high relative humidity and free droplets of water (Khan et al. 2008; Oerke et al. 2019; Tan et al. 2023). After germination, *C. beticola* penetrates stomatal openings, allowing access into plant mesophyll tissue (Steinkamp et al. 1979; Tan et al. 2023). Colonization of sugar beet begins in a biotrophic phase, where it grows in leaf subepidermal tissue, presumably undetected by its host (Steinkamp et al. 1979). As early as five days after infection, *C. beticola* switches to a necrotrophic lifestyle phase characterized by the release of various effector molecules such as cercosporin and beticolin, leading to tissue necrosis (Rangel et al. 2020). The infection manifests as small purple or brown lesions with white or tan interiors (Rangel et al. 2020). As disease progresses, the number and size of these lesions increases, forming large areas of necrotic leaf tissue, which can ultimately lead to the death of infected leaves (Steinkamp et al. 1979). Mature lesions will produce pseudostromata and conidia, allowing the life cycle to repeat multiple times in a single growing season (Rangel et al. 2020). Although a sexual stage has never been definitively identified in *C. beticola*, populations

show strong evidence of sexual recombination which may help to explain its high rate of gene flow and evolution (Groenewald et al. 2007; Bolton et al. 2012b; Bolton et al. 2014).

1.2.2. Yield loss and management of Cercospora leaf spot

C. beticola is the most devastating foliar disease of sugar beet worldwide (Rangel et al. 2020). When CLS is unmanaged, infected sugar beet leaves die, requiring the crop to use stored sugar in the root to replace them. This results in lower recoverable sucrose, which equates to economic losses for growers and sugar refineries (Steinkamp et al. 1979). A loss of up to 50% recoverable sucrose per infected beet has been suggested in severe cases (Shane and Teng 1992). In addition to the loss of total sucrose, impurities accumulate within the roots of diseased plants, further devaluing infected beets (Shane and Teng 1992). Annual economic losses from sugar yield loss, impurities, and the costs of fungicide applications are estimated to exceed several million dollars in the US (Bolton et al. 2016).

Various measures of control have been implemented to help reduce the impact of CLS, including breeding resistance, crop rotation and deep tillage to reduce inoculum load, and the use of fungicides (Rangel et al. 2020). The introduction of genes from wild beet relatives, including *B. vulgaris* spp. *maritima*, has been the primary source of resistance breeding, but this often comes at a cost to sugar yield potential (Munerati 1932; Monteiro et al. 2018). Fungicide application remains one of the most widely used means of controlling potential damage from CLS (Shrestha et al. 2020). Some of the major fungicides used to control CLS include demethylase inhibitors (DMIs), quinone outside inhibitors (QoIs), benzimidazoles, and triphenyltin compounds (Rangel et al. 2020).

1.3. Fungicides

1.3.1. Broad spectrum and site-specific fungicides

Fungicides are chemical compounds designed to kill or inhibit the growth of fungi. They are frequently used in large-scale agriculture to protect or treat crops against fungal colonization (Zubrod et al. 2019). Fungicides may be used to control disease, reduce blemishes, and improve storage quality after harvest (McGrath 2004). Fungicides can be classified based on several different criteria. Based on specificity, fungicides are either broad spectrum (multi-site) or site-specific. Broad spectrum fungicides are characterized by a general toxicity to most fungi and potentially other microbes (Yellareddygari et al. 2019). These fungicides are generally cheaper, and while they help protect against a variety of fungi, they tend to be less effective when crops are faced with heavy disease pressure from particular pathogens (Yellareddygari et al. 2019). Some common broad-spectrum fungicides include chlorothalonil, manzate, dithiocarbamates, and pyrimethanil (Phillips and Hausbeck 2019). In contrast, site-specific fungicides target single, specific loci with high effectiveness. DMIs and QoIs are examples of site-specific fungicides.

1.3.2. Contact and systemic fungicides

Fungicides can be classified as contact or systemic. Contact fungicides remain on the surface of the plant after application, without being absorbed internally by the plant (Bayer. 2021.). These fungicides are usually only effective before pathogen infection and are thus considered to be preventative fungicides (Bayer. 2021.). Because contact fungicides are often broad spectrum, they tend to work against a variety of infectious agents (McGrath 2004). However, since they remain on the surface of the plant, there is a risk of being washed off by rain or irrigation (Bayer. 2021.). Systemic fungicides are absorbed by the plant and can be taken to other tissues within the plant, allowing for the control of disease after infection has started (Bayer. 2021.). Although

systemic fungicides are often considered to be “curative”, their ability to stop or reverse infection is usually limited to very early infections (McGrath 2004). Systemic fungicides are often site-specific, being highly effective against a narrower range of pathogens (Bayer. 2021.). However, systemic fungicides inherently become diluted as they are spread throughout the plant, and may require high doses to be effective, which corresponds to higher cost of application (McGrath 2004; Bayer. 2021.).

1.3.3. Drawbacks of fungicide use

While fungicides can be very effective at mitigating disease on crops, there are some notable drawbacks. Primarily in the case of broad-spectrum fungicides, general toxicity can translate to harmful health effects for human health and the environment, especially in aquatic ecosystems (Zubrod et al. 2019). It can also be very costly to apply fungicides to entire fields, and this money can be wasted if applied at the wrong time. Improper fungicide application can occur for a number of reasons. There may be no disease pressure, and thus no reason to apply fungicides, disease progression may be too advanced and fungicides would be ineffective, or a particular pathogen population may have developed resistance to the fungicide being applied, and dosage applied is too low (Secor et al. 2010; van den Berg et al. 2016). The common progression of fungal pathogens evolving resistance to fungicides is another drawback to relying on these products to manage disease. It has also been widely hypothesized that extensive fungicide application is a driving force behind the development of fungicide resistance in environmental fungal species, which may even carry over to human pathogens (Bastos et al. 2021). Despite the drawbacks, fungicide use remains one of the primary methods of disease management in the United States, and it remains largely effective when the type and timing of fungicide application is well informed by research (Secor et al. 2010).

1.4. FRAC groups

One of the greatest problems associated with the reliance of fungicides to protect crops is the recurrence of fungi developing resistance to these chemicals after repeated use. Fortunately, there are many different compounds targeting a variety of critical cellular functions which are commercially available as fungicides. By rotating fungicides with different modes of action, growers can keep ahead of evolving pathogen resistance (Secor et al. 2010). Often, when pathogens evolve resistance to a fungicide or chemical, there will be fitness penalties such as decreased virulence, slower growth, or decreased spore production (Mikaberidze and McDonald 2015). This means there is often a high cost to maintaining fungicide resistance, so changing the type of fungicide used can remove the pressure to maintain resistance, and thus lead to reduction in resistance levels toward unused fungicides (Secor et al. 2010). To exploit this as an effective management strategy, it is imperative that growers understand which chemicals a pathogen is likely to have cross resistance with.

To help growers understand which fungicides have cross resistance behavior, the Fungicide Resistance Action Committee (FRAC) has established a fungicide classification system grouping chemicals by this metric. This generally means that fungicides are categorized by similarity in chemistry and mode of action. There are several categories that the different groups of fungicides fall into according to target area of the cell. According to the FRAC website, the areas targeted include: nucleic acid metabolism, cytoskeleton and motor proteins, respiration, amino acid and protein synthesis, signal transduction, lipid synthesis or transport/membrane integrity or function, cell wall melanin synthesis, sterol biosynthesis in membranes, cell wall biosynthesis, host plant defense induction, multisite activities, biologicals with multiple modes of action, and unknown mode of action. Each of these categories is given a letter, ranging from A to M. The only two-letter

code, BM, corresponds to biologicals with multiple modes of action. Within each category there are different FRAC groups which are identified in two ways. First, each FRAC group has a code for the target class it belongs to. For example, category A is nucleic acids metabolism, and within this category there are five FRAC groups targeting different aspects of nucleic acid metabolism, which are given the codes A1 through A5. Additionally, each FRAC group has its own unique number, which roughly corresponds to the point in time the group became publicly available. For groups A1 through A5, these numbers are 4, 8, 32, 31, and 52, respectively. Fungicides may be represented by either or both codes (FRAC 2023).

1.5. Demethylation inhibitors and CYP51

1.5.1. Demethylation inhibitors

One widely used group of fungicides are demethylation inhibitors (DMIs). According to Zubrod et al. (2019), DMIs accounted for approximately 7% of total fungicide used in the US in 2016. DMIs have the FRAC identification codes G1 and 3. DMIs can be classified into six different types of molecules. These are piperazines, pyridines, pyrimidines, imidazoles, triazolinthiones, and triazoles. The largest group are triazoles, which include compounds such as tetraconazole and difenoconazole. DMIs are systemic fungicides, with a site-specific mode of action. The target molecule is lanosterol-14α-demethylase, which is coded for by the gene *erg11*, more commonly known as *Cyp51* (FRAC 2023).

1.5.2. CYP51

CYP51 is an enzyme in the cytochrome P450 (CYP) monooxygenase superfamily (Zhang et al. 2019). CYP enzymes are an abundant group of heme-containing enzymes playing important roles in both primary and secondary metabolic pathways in all biological kingdoms (Lepesheva and Waterman 2007; Zhang et al. 2019). Their basic function is the addition of an oxygen atom to

a substrate molecule via the ferrous heme functional group (McDonnell and Dang 2013; Gilani and Cassagnol 2024). In general, an oxygen molecule (O_2) bound to the heme iron is reduced twice by electron transfer from NADPH to create a peroxide state (Gilani and Cassagnol 2024). The peroxide is then doubly protonated resulting in the addition of oxygen to a bound substrate and the release of a water molecule (Gilani and Cassagnol 2024). CYP enzymes are membrane bound, typically associated with the endoplasmic reticulum and inner mitochondrial membrane (Gilani and Cassagnol 2024).

Cyp51 genes are found in all biological kingdoms and represent the most widespread group of CYP molecule (Lepesheva and Waterman 2007, 2011). These enzymes catalyze the oxidative removal of 14α-methyl groups of sterol precursors (Lepesheva and Waterman 2007). The intermediate compounds catalyzed by CYP51 are further processed by other biosynthetic enzymes, leading to the formation of cholesterol in animals, ergosterol in fungi, and various 24-alkylated olefinated sterols in plants, algae and protozoa (Lepesheva and Waterman 2007). In the case of fungi, CYP51 demethylates lanosterol to form 4,4-dimethylcholesta-8,14,24-trienol in the multistep biosynthesis of ergosterol from the precursor of 2 acetyl-coA molecules (Jordá and Puig 2020). Most of the enzymes involved in ergosterol biosynthesis, including CYP51, are located in the endoplasmic reticulum, while some enzymes are located in the cytoplasm (Liu et al. 2019; Zhang et al. 2019). The biosynthesis of ergosterol is a critical process in fungi, as ergosterol is key for membrane fluidity and permeability (Liu et al. 2019; Zhang et al. 2019). In addition to being a necessary membrane component, ergosterol acts as a primary fungal hormone involved in growth and proliferation, maintenance of mitochondrial DNA, and hypoxic and iron deficiency response (Shakoury-Elizeh et al. 2010; Cirigliano et al. 2019; Jordá and Puig 2020). Unsurprisingly, the ergosterol biosynthesis pathway is a common target for fungicides, with molecular classes

including statins, allylamines, DMIs, morpholines, and polyenes blocking or inhibiting various enzymes in this pathway (Jordá and Puig 2020).

1.6. General fungicide resistance

Fungicide resistance is one of the biggest problems facing modern agriculture (Deising et al. 2008). Pathogen resistance to fungicides has been reported across the globe since 1964 and has resulted in the loss of entire classes of fungicides as means of control against some pathogens (Anderson 2005; Deising et al. 2008). Resistance can be categorized as either qualitative or quantitative (Deising et al. 2008; Taylor and Cunniffe 2023). Qualitative resistance refers to resistance incurred by genetic modification of a single major gene (Deising et al. 2008; McGrath 2020). This usually results in complete resistance, and higher doses of fungicide do not help recover disease control (McGrath 2020). For example, when quinone outside inhibitors (QoIs) were first introduced in the 1990s, they had great efficacy against wheat pathogen *Zymoseptoria tritici,* but became ineffective after a few years due to a G143A amino acid substitution in the cytochrome b target site (Blake et al. 2018; Taylor and Cunniffe 2023). Qualitative resistance represents dramatic shifts in fungicide efficacy, which translates to substantial economic losses due to application of ineffective fungicides, as well as yield losses (Secor et al. 2010; McGrath 2020). In contrast, quantitative resistance results from the accumulation of multiple genetic changes, which make minor contributions to overall resistance (McGrath 2020; Pereira et al. 2020). Quantitative resistance gradually increases over time, and higher doses of fungicides are required to achieve proportional inhibition (McGrath 2020). An example of quantitative resistance can be seen in cucurbit powdery mildew, primarily caused by *Podosphaera xanthii* (McGrath 2020; Schuh and Grabowski 2022). This pathogen has become fully resistant to the first

commercially introduced DMI fungicide, Bayleton, but is still sensitive to other DMI fungicides with higher efficacy (McGrath 2020).

1.7. DMI Resistance

1.7.1. non-*Cyp51* related DMI resistance

Multiple mechanisms unrelated to *Cyp51* have been documented to impact DMI resistance in fungi. Taken together, these could help to explain possible incremental changes toward the development of quantitative resistance. One such mechanism is the overexpression of membrane-bound efflux pumps and ATP-binding cassette (ABC) transporters (Cools and Fraaije 2013). In *Z. tritici*, overexpression of *MFS1* encoding a drug efflux transporter was enabled by a 519-bp long terminal repeat retrotransposon insertion into the *MFS1* promoter (Omrane et al. 2017). DMI resistance in *Z. tritici* was also associated with pleiotropic effects caused by PKS1, a polyketide synthase involved in the synthesis of dihydroxynaphthalene melanin, in a population study by Lendenmann et al (2015). The study found the nucleotide position 1783 of *PKS1* to be the source of phenotypic variance for melanization, growth rate in the presence and absence of fungicide, and fungicide resistance (Lendenmann et al. 2015). A study by Zhang et al. (2020) identified 19 differentially expressed genes upregulated in a DMI resistant *Penicillium italicum* strain in response to treatment with the DMI prochloraz. Both RNA sequencing and quantitative real time polymerase chain reaction (qPCR) confirmed the upregulation of these genes, which included ABC transporter genes, major facilitator superfamily (MFS) transporter genes, ergosterol anabolism genes *Erg2* and *Erg6*, mitogen-activated protein kinase (MAPK) signaling-inducer genes *Mkk1* and *Hog1*, and Ca_2^+/calmodulin-dependent kinase (CaMK) signaling inducer genes *CaMK1* and *CaMK2* (Zhang et al. 2020). Finally, other genes implicated in DMI resistance remain uncharacterized (Ballard et al. 2019). A study by Ballard et al 2019 found a strain of *A. fumigatus*

carrying a single nucleotide polymorphism (SNP) resulting in a nonsense mutation in an uncharacterized gene had elevated resistance to multiple azole DMIs (Ballard et al. 2019). By introducing this mutation into a different azole-susceptible strain using CRISPR-Cas9, elevated resistance to itraconazole was obtained (Ballard et al. 2019).

1.7.2. *Cyp51*-related DMI resistance

DMI resistance linked to *Cyp51* is well documented. This manifests primarily as mutations within the coding sequence of *Cyp51*, overexpression of *Cyp51*, or copy number variation. The principal way in which *Cyp51* overexpression has been reported to occur is by the insertion of enhancer sequences into the promoter sequence of *Cyp51* (de Ramón-Carbonell and Sánchez-Torres 2020). In *Penicillium digitatum*, strains with five copies of a native enhancer in the promotor region have increased resistance to the DMI imazalil compared with strains containing one copy (Hamamoto et al. 2000; Ghosoph et al. 2007). Additionally, strains which have one copy of the enhancer plus an additional 199bp insert also had increased resistance (Ghosoph et al. 2007; Sun et al. 2011). Both cases of resistant strains exhibited significant overexpression of *Cyp51* above the sensitive strains (Ghosoph et al. 2007). Similar examples of increased DMI resistance associated with promoter insertions have been reported in other fungal pathogens, including *Monilinia fructicola, Venturia inaequalis, Blumeriella jaapii,* and *Z. tritici* (Schnabel and Jones 2001; Ma et al. 2006; Luo et al. 2008; Leroux and Walker 2011; de Ramón-Carbonell and Sánchez-Torres 2020). Aside from promoter insertions, examples of DMI resistance correlated with *Cyp51* overexpression with uncharacterized mechanisms have been reported in multiple species, including *Lasiodiplodia theobromae, Pyrenophora teres, Colletotrichum gloeosporiodies,* and *Botrytis cinerea* (Mair et al. 2016; Wei et al. 2020; Wang et al. 2021; Zhang et al. 2021).

Another means of *Cyp51*-related DMI resistance occurs in *Cyp51* copy number variation. Up to four different paralogues of *Cyp51* have been identified in fungi, with recent evidence suggesting three of these (*Cyp51A*, *Cyp51B*, and *Cyp51C*) diverged within the ascomycete subdivision pezizomycetes, which contains most filamentous ascomycetes (Celia-Sanchez et al. 2022). While it is believed that all ascomycetes have the functional paralogue *Cyp51B*, paralogue *Cyp51A* is found in only some ascomycetes, while *Cyp51C* is found primarily in *Fusarium* species (Chen et al. 2020; Celia-Sanchez et al. 2022). A study by (Chen et al. 2020) found evidence that the paralogues *Cyp51A* and *Cyp51B* in *Colletotrichum* species were differentially affected by different DMIs (Chen et al. 2020). Namely, that tebuconazole showed a higher binding affinity to *Cyp51A* while prothioconazole, cyproconazole, difenoconazole, and prochloraz appeared to primarily target *Cyp51B* in *Colletrotrichum fioriniae* (Chen et al. 2020). This preferential targeting of different *Cyp51* enzymes suggests that having multiple *Cyp51* variants may be advantageous toward ensuring the continued function of at least one variant under DMI pressure.

1.7.3. *Cyp51* mutations associated DMI resistance

Mutations in *Cyp51* are a widely reported and well-studied indicator of DMI resistance. These mutations often occur in highly conserved regions of CYP51, such as substrate recognition sites (SRS). The vast majority of mutations in *Cyp51* associated with DMI resistance are nonsynonymous, although evidence for synonymous mutations (which do not represent a change in amino acid sequence) impacting DMI resistance has been established (Spanner et al. 2021). The nonsynonymous mutation Y123H in *Cyp51B* in *Fusarium graminearum* has been demonstrated by site-directed mutagenesis to reduce prochloraz sensitivity, among other effects including conidiation and ascospore development (Zhao et al. 2021). The tyrosine in position 123 of *F. graminearum* is highly conserved across phyla, and mutations at equivalent positions in other

fungal species have likewise been associated with DMI resistance (Zhao et al. 2021). Another commonly reported location can be represented by the mutations Y132F, in *Candida albicans*, Y144H/F in *Parastagonospora nodorum*, and Y137H in *Z. tritici* (Cools and Fraaije 2013; Pereira et al. 2017; Sagatova et al. 2018). These two loci are found within the highly conserved SRS 1, a short amino acid sequence implicated in substrate binding (Lepesheva and Waterman 2007; Zhang et al. 2019). Numerous mutations have also been reported within the heme-binding pocket sequences. Some examples of these mutations include G448S, G464S, and G484S in *A. fumigatus*, *C. albicans*, and *Cryptococcus neoformans*, respectively (Rodero et al. 2003; Pelaez et al. 2012; Sagatova et al. 2018). These are equivalent mutations associated with resistance to short-tailed triazoles in human pathogens (Sagatova et al. 2018).

While qualitative resistance is generally considered to be conferred by target-site mutations, not all mutations of *Cyp51* affect DMI resistance at the same level. Some mutations are very strongly correlated with resistance. In a study which investigated overexpression and mutations of *Cyp51* as causes of DMI resistance in *P. teres* f. sp. *teres*, the mutation F489L was found to be only present in DMI resistant isolates, and all resistant isolates carried the mutation (Mair et al. 2016). Some mutations seem to only have an effect in combination with other mutations (Sanglard et al. 1998). Mutant strains of *Saccharomyces cerevisiae* which received the mutation G464S showed no difference in azole sensitivity, but when coupled with the Y140F mutation exhibited 4-fold reduction in sensitivity to fluconazole (Sagatova et al. 2018). Still, there are other mutations seen within *Cyp51* that have not been associated with DMI resistance (Cools and Fraaije 2013).

1.8. DMI Resistance in *C. beticola*

Both overexpression and mutations of *CbCyp51* have been correlated with DMI resistance in *C. beticola*. DMI resistant isolates from Northern Greece have been found to display up to a 234-fold increase in *CbCyp51* expression (Nikou et al. 2009). Overexpression has subsequently been identified in DMI resistant strains in the RRV and the Czech Republic (Bolton et al. 2012a; Kumar et al. 2021). Various *CbCyp51* mutations have been shown to have influence on DMI resistance in *C. beticola*. Phenotypes exhibiting high levels of resistance in European field isolates have been linked to the mutations Y464S, I309T+L144F, and L144F (Muellender et al. 2021). The L144F mutation has also been linked to DMI resistance in other areas across the globe, including Japan and the RRV (Kayamori et al. 2021; Spanner et al. 2021). Interestingly, recent evidence has suggested synonymous mutations play a role in DMI resistance. From two independent studies in Greece and the RRV, mutation in the wobble position of glutamic acid codons E169 and E170, respectively, from guanine to adenine have been associated with high resistance to DMIs (Nikou et al. 2009; Spanner et al. 2021). Other mutations discovered in moderately and highly resistant isolates from N. Greece include E297K, I330T, and P384S (Nikou et al. 2009).

1.8.1. DMI resistance associated *CbCyp51* mutations in RRV *C. beticola* populations

A recent genome-wide association (GWAS) study on RRV isolates identified correlations between L144F, E170, and the combination L144F+E170, and tetraconazole resistance (Spanner et al. 2021). Rangel et al. (unpublished data) has expanded on this work, correlating differential sensitivity to various DMI fungicides among 2021 RRV isolates representing five *CbCyp51* haplotypes, differing at the amino acid positions L144 and E170. Two variants of the mutation L144F, corresponding to codons TTC (L144F_C) and TTT (L144F_T), show differential sensitivity to DMIs. Isolates containing the TTC codon were more likely to be resistant to the

DMIs tetraconazole (Tet), prothioconazole (Pro), difenoconazole (Dif), and mefentrifluconazole (Mef), while those containing the TTT codon were more likely to be sensitive to all four DMIs. Interestingly, isolates with the mutation L144F_T were more likely to be sensitive to DMIs than isolates with the 'wild-type' *CbCyp51* haplotype, which contains no mutations. Furthermore, the amino acid E170 was associated with DMI resistance based on the codon used. Generally, isolates containing the codon GAG (E170_G) correlate with DMI sensitivity, while those containing the codon GAA (E170_A) correlate with DMI resistance. However, specifically which DMIs the E170 mutation predicts resistance to depends upon the presence or absence of the L144F_C mutation. When the E170 mutation was found alone, it strongly predicted resistance to Tet and Pro (with at least 97.5% accuracy), but strongly predicted sensitivity to Dif and Mef (with at least 92.6% accuracy). When E170_A was found in conjunction with the L144F_C, this haplotype strongly predicted resistance to all four fungicides, at higher percentage than with L144F_C alone. Interestingly, out of nearly 600 isolates from the 2021 RRV population used in Rangel et al study, none were found that contained the combination of E170_A with L144F_T, which may indicate a fitness penalty for this combination. Thus, the five major haplotypes of *CbCyp51* based on these two amino acid positions represented in the RRV are L144+E170_G, L144F_T+E170_G, L144F_C+E170G, L144+E170_A, and L144F_C+E170_A. These haplotypes will be referred to by the letters A, B, C, D, and E, respectively (Table 1). According to Rangel et al, the distribution of haplotypes in the 2021 RRV population out of 593 isolates are as follows: 80 isolates of haplotype A (13.5%), 82 isolates of haplotype B (13.8%), 19 isolates of haplotype C (3.2%), 122 (20.6%) isolates of haplotype D, and 290 isolates of haplotype E (48.9%). Haplotype E, which is the strongest predictor of resistance to all four DMIs tested (with over 90% accuracy to each), is by far the most abundant isolate sampled from 2021, which suggests selective pressure on *C.*

beticola by DMI usage. Of perhaps the greatest intrigue among the findings of Rangel et al. (unpublished data) and Spanner et al. (2021) is the stark difference in DMI sensitivity prediction based on synonymous mutations in *CbCyp51*.

Effects of nonsynonymous mutations are better understood in *CbCyp51*. The L144F substitution occurs within SRS 1, and likely influences binding of DMI compounds at this location. However, for synonymous mutations, phenotypic changes cannot be influenced by enzyme structure. Potential explanations include altered chromatin structure and recognition by DNA and RNA regulatory elements (Plotkin and Kudla 2011; Zhou et al. 2016) as well as the availability of tRNAs corresponding to each codon variant (Liu et al. 2021). However, the specific reasons for synonymous mutations influencing DMI resistance in *C. beticola* have yet to be elucidated.

2. CHAPTER TWO: EFFECTS OF SYNONYMOUS AND NONSYNONYMOUS *CYP51* MUTATIONS ON DMI RESISTANCE IN *CERCOSPORA BETICOLA*

2.1. Abstract

Cercospora beticola is the most devastating foliar pathogen of sugar beet. This pathogen is primarily managed by the application of fungicides, including demethylation inhibitors (DMIs). Given the broad use of DMIs in managing Cercospora leaf spot, resistance has developed. Recently, five haplotypes of *CbCyp51* have been correlated with DMI resistance. To improve our understanding of these haplotypes, this study has evaluated *CbCyp51* expression across haplotypes with and without DMI exposure using RT-qPCR. Significant differences were found between haplotypes in control and difenoconazole groups, but the broader implications were unclear. This study also produced mutant *C. beticola* strains with replaced *CbCyp51* haplotypes. DMI sensitivity was assessed, revealing dramatic changes in difenoconazole resistance. Most mutants exhibited elevated tetraconazole resistance, which was largely shown to be associated with the transformation process rather than haplotype exchange. Further studies involving the different haplotypes and mutants could improve our understanding of these DMI resistance associated mutations.

2.2. Introduction

Cercospora beticola is the most devastating foliar disease of sugar beet worldwide. Common management practices like deep tillage and crop rotation help reduce inoculum load. However, these methods do not provide total control, and the most common strategy used to minimize *C. beticola* infection in sugar beet is the timely application of fungicides. Common fungicides used in CLS management include DMIs, QoIs, benzimidazoles, and triphenyltin compounds.

The efficacy of fungicides has been impacted by the development of resistance in *C. beticola* populations. Resistance has developed in every major class of fungicide employed. The first fungicides used against *C. beticola* in the RRV were benzimidazoles, implemented in the 1970s after the introduction of sugar beet varieties with higher sucrose yield but greater susceptibility to CLS (Secor et al. 2010). The rapid development of resistance to this class of fungicides resulted in a CLS epidemic in 1981, at which point most growers switched to the use of triphenyltin hydroxide (Secor et al. 2010). This remained the primary fungicide applied to manage CLS until resistance began developing in the mid-1990s. Following an outbreak of CLS in 1998 which caused an estimated $45 million in losses due to reduced yield and fungicide application costs, tetraconazole was approved for emergency use as the first DMI fungicide against *C. beticola* (Secor et al. 2010; Bolton et al. 2016). QoIs were also introduced in 2002, but widespread resistance has developed to this class of fungicide as well, primarily correlated with a highly prevalent G143A amino acid substitution in the cytochrome b gene (Secor et al. 2010; Bolton et al. 2013; Rangel et al. 2020). Despite resistance, fungicides remain the most effective control method against CLS, and a rotation of DMIs and QoIs are currently used to manage the disease.

Presently, there are only a few strategies used to help maintain the efficacy of fungicides. One strategy is the mixing of systemic fungicides, such as DMIs, with contact fungicides to create a combined higher efficacy of control (Rangel et al. 2020). The other strategy involves fungicide rotation, both within and between seasons (Secor et al. 2010). Fungicide rotation helps reduce the pressure on *C. beticola* to gain resistance to any one class since the inhibitory molecule is ever-changing (Secor et al. 2010). For fungicide rotation to work effectively, accurate and current knowledge of fungicide resistance levels in *C. beticola* field isolates is necessary. To assist in

forecasting fungicide resistance, PCR tests have been successfully demonstrated to quickly detect the presence of resistance-associated point mutations in *C. beticola* (Shrestha et al. 2020).

In the case of DMIs, the combination of mutations located at positions L144 and E170 of *CbCyp51* constitute five haplotypes that have varying degrees of accuracy predicting sensitivity or resistance to different triazoles (Rangel et al, unpublished data). Conducting studies on the nature of mutation-related resistance could help improve our understanding of the role of these mutations in resistance to DMIs, which in turn allows us to be better informed about DMI resistance based on the presence or absence of molecular markers.

Previous studies on *CbCyp51* expression have found a correlation between higher tetraconazole tolerance and elevated basal level and induced expression of *CbCyp51* (Bolton et al. 2012a; Bolton et al. 2016). During the time of these studies, no mutations within *CbCyp51* had been correlated to DMI resistance in RRV populations (Bolton et al. 2012a). Since the discovery of the resistance trends seen in the previously defined *CbCyp51* haplotypes, the question of whether or not these haplotypes are correlated to different levels of *CbCyp51* expression has remained unstudied. To investigate this question, we have analyzed three isolates of each *CbCyp51* haplotype of *C. beticola* collected in the RRV in 2021 for *CbCyp51* expression with and without DMI exposure. In addition, to further confirm the direct effects of the L144F and E170 mutations, RRV isolates were mutated to change native *CbCyp51* haplotypes, and half maximal effective concentrations (EC_{50}) of DMI inhibition of radial growth were determined by DMI resistance assays to assess if *CbCyp51* replacements affected fungicide sensitivity.

2.3. Materials and methods

2.3.1. *CbCyp51* expression analysis

CbCyp51 gene expression was quantified using a strategy similar to Bolton et al. (2012a) where *CbCyp51* gene expression in isolates were compared with a single calibrator isolate with high sensitivity to Tet and Dif (haplotype A). Strains were chosen from isolates collected in the Red River Valley in 2021 (provided by Gary Secor, NDSU). Three isolates of each haplotype were selected with EC_{50} values close to the median population EC_{50} value for both tetraconazole and difenoconazole. The following isolates were used: Haplotype A (21-574, 21-384, 21-354; median Tet=5.68, Dif=0.72), Haplotype B (21-150, 21-536, 21-540; median Tet=2.97, Dif=0.89), Haplotype C (21-493, 21-569, 21-222; median Tet=43.24, Dif=40.00), Haplotype D (21-227, 21-443, 21-532; median Tet=43.79, Dif=1.00), and Haplotype E (21-245, 21-258, 21-502; median Tet=57.69, Dif=68.84) were used (Rangel et al. unpublished data). Haplotype A isolate 21-354 had an amino acid substitution I387M; no other isolates used had any additional *CbCyp51* mutations.

Table 1. *CbCyp51* haplotypes with predicted resistance (R) or sensitivity (S) to Tetraconazole (Tet) and Difenoconazole (Dif). Codons followed by (F) indicate an amino acid change to phenylalanine.

Haplotype	L144 codon	E170 codon	Tet Prediction	Dif Prediction
A	TTG	GAG	S	S
B	TTT (F)	GAG	S	S
C	TTC (F)	GAG	R	R
D	TTG	GAA	R	S
E	TTC (F)	GAA	R	R

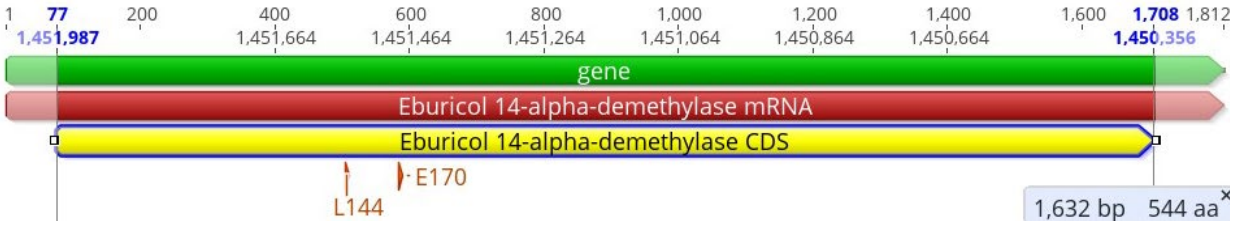


Figure 1. Representation of CbCyp51 gene, mRNA, and coding sequence (CDS) using Geneious Prime software v. 2024.0.2. Positions of L144 and E170 are marked. Top numbering indicates nucleotide position of the gene. Bottom numbering indicates nucleotide position within the chromosome context.

2.3.2. *CbCyp51* expression study fungal tissue growth and harvest

To initiate growth, 5 mm plugs from the original isolate plates were placed on potato dextrose agar (PDA) plates (BD Diagnostics, Franklin Lakes, NJ, USA). These plates were grown in the dark at 22°C for 21 days. Agar plugs from these plates were used to start liquid cultures for Tet and Dif treatment groups at 14 days and 21 days, respectively. For each treatment group, six liquid cultures for each isolate were initiated by placing agar plugs taken from the leading edge of the PDA cultures into 50-ml conical tubes (Corning, Glendale, AZ, USA) containing 17.5 ml of potato dextrose broth (PDB) (BD Diagnostics, Franklin Lakes, NJ, USA). Isolates were allowed to grow for 96 h in total darkness at 25°C shaking at 100 rpm in a MaxQ 420 HP incubator (Thermofisher Scientific, Waltham, MA, USA) with tubes oriented horizontally, placed in a random order between 4 tube racks. After this time, a 17.5-µl spike of either acetone (3 tubes) or DMI fungicide in acetone at 10,000 µg ml^{-1} (for a final concentration of 10 µg ml^{-1}, 3 tubes) was added and all samples were returned to incubation. After 48 hours, mycelium was harvested by pouring sample contents through 2 layers of miracloth (MilliporeSigma, Burlington, MA, USA) and immediately frozen in liquid nitrogen. Samples were stored at -80°C until homogenization in liquid nitrogen using mortar and pestle.

2.3.3. RNA extraction, cDNA synthesis, and RT-qPCR

RNeasy Plus Mini kit (Qiagen, Germantown, MD, USA) was used to isolate total RNA following the manufacturer's instructions. RNA samples were DNase treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) before cDNA synthesis, following the manufacturer's instructions. Total RNA (100 ng) was used for cDNA synthesis using a Super-Script IV reverse transcriptase kit (Invitrogen, Waltham, MA, USA) according to the manufacturer's instructions. Amplification was performed using oligo-(dT) primers from the reverse transcriptase kit. The resulting cDNA was used as a qPCR template. The qPCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). Plates were run on a C1000 Touch thermal cycler (Bio-Rad, Hercules, CA, USA) equipped with a CFX96 Real-Time PCR Detector (Bio-Rad, Hercules, CA, USA). Real-time PCR conditions were as follows: an initial 95°C denaturation step for 2 minutes followed by denaturation for 5 seconds at 95°C, annealing and extension for 30 seconds at 60°C, followed by a plate read for a total of 40 cycles. No-reverse-transcriptase (NRT) and no-template (NT) controls were included for every qPCR run.

2.3.4. Expression data processing

CbCyp51 expression was quantified with primers MDB-2250 and MDB-2288 (Table 2) using the Pfaffl method (Pfaffl 2001) with actin as a reference gene. The primers used for actin amplification were MDB-284 and-MDB 285 (Table 2). Amplification efficiencies for the primer pairs MDB-2250/MDB2288 and MDB-284/MDB-285 were 1.942 and 1.837, respectively. CFX Maestro analysis software (version 1.1; Bio-Rad, Hercules, CA, USA) was used to visualize amplification curves and acquire cycle threshold values. X-fold expression values were calculated by dividing induced expression values by basal expression values. All expression values were compared to the sensitive haplotype A isolate 574 basal expression. A one-way analysis of variance

(ANOVA) ($p \leq 0.05$) was performed to determine statistical differences in basal expression, induced expression, and X-fold change across haplotypes for each trial. For each comparison that showed a significant difference between haplotype means, a Tukey HSD test was used to identify significant differences between each pair-wise mean comparison of haplotypes ($p \leq 0.05$).

Table 2. Primers used in this study.

Primer	Name	Sequence (5' to 3')
MDB-258	Split-marker: HY (NLC37)	GGATGCCTCCGCTCGAAGTA
MDB-259	Split-marker: YG (NLC38)	CGTTGCAAGACCTGCCTGAA
MDB-284	C.beticola actin qPCR For	ACATGGCTGGTCGTGATTTG
MDB-285	C.beticola actin qPCR Rev	TGTCCGTCAGGAAGCTCGTA
MDB-392	*Cyp51*-upstream_Rev	AAGGCACTCTGTATCGATGG
MDB-2288	L144F F3	AACTGCGCGATGTCAATG
MDB-2250	*Cyp51* R	TCGCTTATGTGGGTTGTTCTT
MDB-3435	*Cyp51* 5' Flank Sequencing	AGGCAATGACGCACGGTCCT
MDB-2373	*Cyp51* L144F sen-1	HEX-CCCCAACTCCAAATTGATGG-Ibfq
MDB-2284	*Cyp51* L144F [C]-1	FAM-CCCCAACTCCAAATT**C**ATGG
MDB-2445	*Cyp51* L144F [T]-15	Texas Red-CCCCAACTCCAAATT**T**ATGGAG-IBRQ-Sp
MDB-2257	E170 Sen-probes-2 (R)	6FAM-AATTGGCGCGT**C**TCTTCG
MDB-2258	E170 Res-probes-2 (R)	HEX-AATTGGCGCGT**T**TCTTCG
MDB-3345	*Cyp51*:pOSCAR 5' check	GCACTTTCTGGTCTCTCGCC
MDB-3346	*Cyp51*:pOSCAR 3' check	CGCGAGACAGAAGAAGCAGC

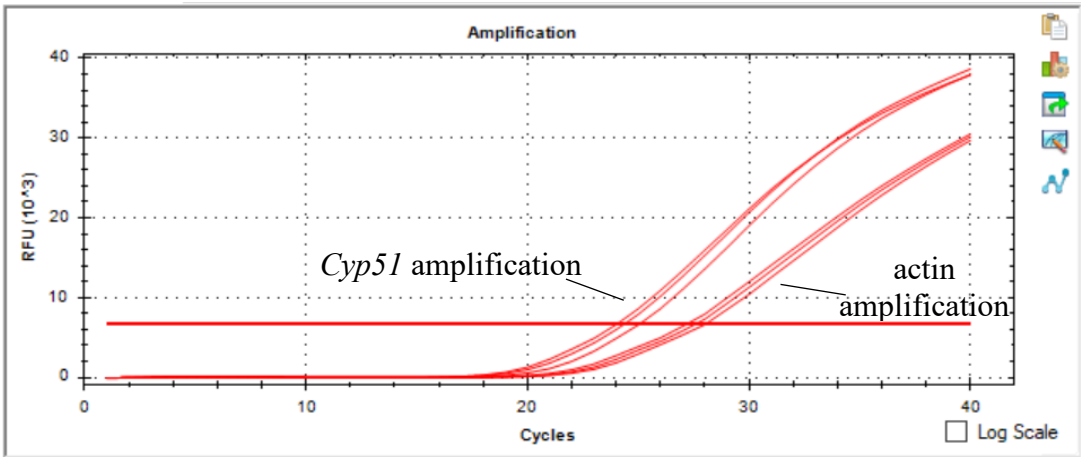


Figure 2. Example qPCR curves for amplification of *CbCyp51* and actin with automatically calculated threshold. (CFX Maestro software v. 1.1)

2.3.5. *CbCyp51* transformation construct

To change the haplotypes of 2021 RRV isolates, a gene replacement construct was developed for use in PEG-mediated transformations. This construct was designed with the entire sequence of *CbCyp51*, followed by a hygromycin B resistance cassette, followed by 574 bp 3' downstream of *CbCyp51* to aid in homologous recombination. The construct was flanked by *SwaI* restriction sites interior to attB sites for potential use in gateway vectors, which were flanked by PacI restriction sites. The gene construct was synthesized in a puc57 vector, which contained an ampicillin resistance cassette for bacterial selection. Identical transformation vectors differing only at the positions L144 and E170 were ordered through GenScript (Piscataway, NJ, USA). The combinations of mutations present in each vector ordered can be found in Table 1. Dehydrated vectors were resuspended in 100 µl dH_2O once received.

Each transformation vector was transformed into One Shot OmniMAX 2 T1 chemically competent *E. coli* cells following the manufacturer's instructions (Invitrogen, Waltham, MA, USA) and plated onto media containing 100 µg ml^{-1} ampicillin (Sigma-Aldrich, St. Louis, MO, USA). To verify cells that had the transformation vector, two fragments covering the hygromycin resistance cassette and part of the 3' flanking region of *CbCyp51* were amplified by PCR. Products were detected on a 1% agarose gel with SYBR safe dye for visualization (APExBIO, Houston, TX, USA). After confirmation of expected bands, *E. coli* colonies containing the transformation vectors were grown in 200 ml superbroth (Fuller and King 1981) with 100 µg ml^{-1} ampicillin (Sigma-Aldrich, St. Louis, MO, USA) to obtain sufficient plasmid quantity for use in transformation. Plasmids were extracted from the cultures using a modified alkaline lysis method (Sambrook and Russell 2006). Briefly, cells were pelleted by centrifugation, resuspended in Tris-EDTA (TE) buffer, lysed with sodium dodecyl sulfate-NaOH buffer, neutralized with potassium

acetate buffer, treated with isopropanol and RNAse-A enzyme (New England Biolabs, Ipswich, MA, USA), washed with 95% ethanol, and resuspended in TE buffer. All plasmids were sequenced using nanopore sequencing through Plasmidsaurus (Eugene, OR, USA) to verify correct sequence. Prior to use, transformation constructs were excised from their vectors with *SwaI* restriction enzyme, following the manufacturer's protocol (New England Biolabs, Ipswich, MA, USA).

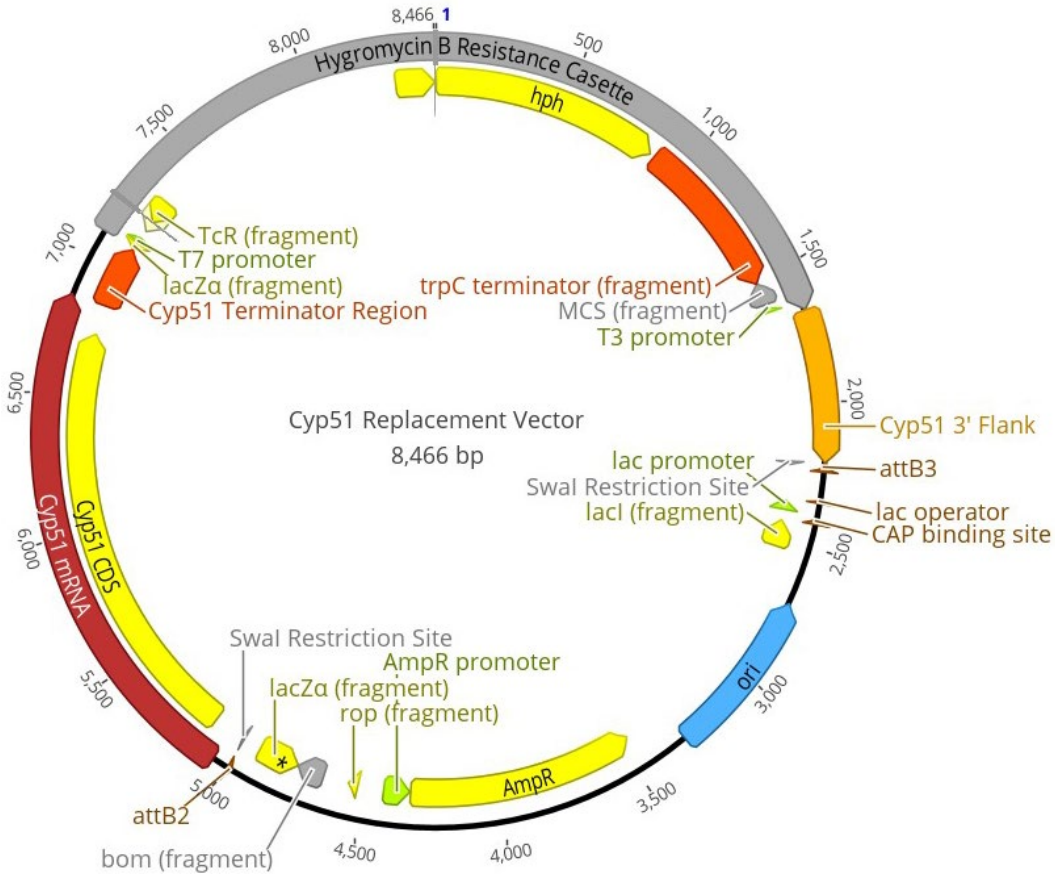


Figure 3. Map of *CbCyp51* replacement vector obtained from Geneious Prime software v. 2024.0.2.

2.3.6. Fungal transformation

The following transformations were performed to change the *CbCyp51* haplotype in 2021 RRV isolates: haplotype E to A (isolate 21-611), haplotype E to B (isolate 21-611), and haplotype D to E (isolates 21-503 and 21-532). PEG-mediated transformations were performed to generate fungal protoplasts from mycelium cultures as described by Liu and Friesen (2012). Protoplasts

were incubated with 30 to 40 µg of transformation construct DNA. Hygromycin B (Thermo Fisher Scientific, Waltham, MA, USA) was added to the regeneration agar at a concentration of 150 µg ml^{-1} for selection. This was either included in the initial plating agar or added as a selective overlay the next day. For each strain, a control culture was included which received no DNA and was plated without hygromycin B (no-DNA control).

2.3.7. Mutant verification and *CbCyp51* copy number assessment

Colonies emerged on the agar surface five to seven days after transformation. Colonies were transferred to PDA amended with 150 µg ml^{-1} hygromycin B (Thermo Fisher Scientific, Waltham, MA, USA). After seven days, a small strip of tissue (~50 mg) was taken from each transformant plate for DNA extraction. Genomic DNA was extracted using the Monarch gDNA kit (New England Biolabs, Ipswich, MA, USA). Transformants that had the gene replacement event were identified using qPCR probes specific to the mutations (Table 1). Probes were combined with qPCRBio Probe Mix (PCRBiosystems, Wayne, PA, USA) for analysis. To isolate genetically pure individuals, single spore colonies were then established for all identified true mutants as well as two or three ectopic mutants and two no-DNA controls. Briefly, small strips of mycelia were taken from each transformant plate and homogenized by adding one sterile 4.5mm BB (Daisy, Arkansas, USA) to 2 ml sample tubes and shaking in a grinder (1600 miniG Spex Sample prep, Metuchen, NJ, USA) shaker. Homogenized tissue was spread on V8 (Campbell's Soup Company, Camden, NJ, USA) media and grown for 4 days at 18°C. After 4 days, spores were transferred to H_2O agar plates by triturating using a tween H_2O solution. The next day, single germinating spores were excised from the plate and transferred to PDA amended with 150 µg ml^{-1} hygromycin B (Thermo Fisher Scientific, Waltham, MA, USA). After single-spore colonies were obtained, the expected *CbCyp51* sequences were verified by sequencing the entire gene including

100 bp upstream and downstream using primers MDB-3435 and MDB-392 (Table 1). Sequencing was carried out through Plasmidsaurus (Eugene, OR, USA). To determine the number of *CbCyp51* insertions in transformants, qPCR was used to measure amplification of *CbCyp51* compared to a standard curve created from two-fold dilutions of a wild-type *CbCyp51* DNA sample (isolate 309-10). After mutant verification, three PDA plates were started from each single spored mutant and no-DNA control plate. DMI resistance assays were initiated from each of the three plates for both tetraconazole and difenoconazole.

2.3.8. DMI resistance assays

DMI resistance assays were carried out as described by Secor and Rivera (2012) for tetraconazole (MilliporeSigma, Burlington, MA, USA) and difenoconazole (MilliporeSigma, Burlington, MA, USA). Five-millimeter agar plugs were removed from the leading edge of each plate and placed on PDA amended with 0.01, 0.1, 1, and 10 μg ml^{-1} DMI, as well as a non-amended control plate. Plates were grown at 22°C in the dark for 15 days. Radial growth measurements were then taken for each plate, averaging the diameter from two perpendicular measurements when possible. Measurement data was used to calculate percentage growth reduction by comparing radial growth on amended media with growth on nonamended media. Percentage growth reduction vs DMI concentration was plotted on a logarithmic scale, and a linear regression curve of best fit was calculated in Microsoft Excel to approximate each EC_{50} value. The control plate was not included in the curve. Resistant/sensitive cutoff values were determined by Rangel et al. (unpublished data), based on the population set. The EC_{50} resistance threshold was 7.58 μg ml^{-1} for Tet, and 5.51 μg ml^{-1} for Dif.

2.4. Results

2.4.1. *CbCyp51* expression results

Cyp51 haplotypes based on the presence/absence of various point mutations in *C. beticola* have been correlated with varying levels of resistance. To determine whether or not *CbCyp51* expression varies by haplotype, an expression study on liquid cultures of *C. beticola* exposed to two different DMI fungicides was carried out. qPCR analysis measured expression levels of *CbCyp51* compared to actin for all samples, measuring controls and treatments across the five haplotypes. All isolates exhibited increased expression of *CbCyp51* after induction with either tetraconazole (Tet) or difenoconazole (Dif) (Figure 1). Expression values for each isolate are located in Table 3. Relative expression calculations revealed significant differences across haplotypes in X-fold expression for the difenoconazole treatment group, and in the control groups of both trials. (Table 4). No significant differences were observed between haplotypes after tetraconazole exposure. Haplotype B isolates had the highest average basal level *CbCyp51* expression in both trials. Haplotype D isolates had the lowest average basal level *CbCyp51* expression in both trials.

Table 3. Basal and Tetraconazole (Tet)- or Difenoconazole (Dif)-induced expression of *CbCyp51* in three isolates of each C. beticola Haplotypes A-E. Expression values are presented relative to Haplotype A isolate 574 basal level expression for each trial (top row). Haplotype A isolate 354 has the I387M mutation and is denoted by an asterisk*. Error values represent standard deviation between biological replicates. Basal and Dif/Tet induced expression were calculated by Pfaffl method (Pfaffl 2001). X-fold expression values were calculated by multiplying induced expression values by basal expression values.

Haplotype	Isolate	Basal Exp Tet Trial	Tet Induced Expression	Tet X-fold Expression	Basal Exp Dif Trial	Dif Induced Expression	Dif X-fold Expression
A E170G+L144G	574	1.00 ± 0.14	8.35 ± 3.81	8.35 ± 3.81	1.00 ± 0.20	12.06 ± 3.16	12.06 ± 3.16
	384	0.72 ± 0.04	11.72 ± 4.76	16.28 ± 6.62	0.21 ± 0.03	1.84 ± 0.92	8.63 ± 4.34
	354*	0.64 ± 0.06	10.45 ± 1.56	15.69 ± 2.34	0.58 ± 0.12	7.53 ± 1.69	12.97 ± 2.92
B E170G+L144FT	150	1.29 ± 0.24	12.13 ± 8.14	9.40 ± 6.31	1.34 ± 0.18	8.69 ± 0.45	6.51 ± 0.34
	536	1.44 ± 0.26	18.73 ± 1.45	13.02 ± 1.01	1.14 ± 0.04	1.29 ± 0.12	1.13 ± 0.10
	540	1.38 ± 0.56	2.64 ± 1.83	1.92 ± 1.33	1.32 ± 0.18	5.69 ± 2.22	4.30 ± 1.68
C E170G+L144FC	222	1.10 ± 0.08	9.15 ± 2.77	8.29 ± 2.51	0.51 ± 0.19	1.72 ± 0.37	3.37 ± 0.72
	493	1.39 ± 0.06	8.96 ± 1.28	6.46 ± 0.92	0.52 ± 0.23	0.62 ± 0.32	1.18 ± 0.61
	569	1.25 ± 0.29	8.35 ± 4.14	6.69 ± 3.32	0.27 ± 0.04	0.93 ± 0.36	3.46 ± 1.35
D E170A+L144G	227	0.50 ± 0.03	7.88 ± 5.61	15.92 ± 11.34	0.38 ± 0.20	2.71 ± 0.89	7.07 ± 2.31
	443	0.64 ± 0.26	8.01 ± 2.54	12.59 ± 3.99	0.32 ± 0.02	6.02 ± 1.13	18.99 ± 3.56
	532	0.69 ± 0.13	6.67 ± 3.13	9.69 ± 4.55	0.34 ± 0.16	5.35 ± 1.71	15.91 ± 5.07
E E170A+L144FC	245	0.77 ± 0.15	3.70 ± 0.94	4.78 ± 1.22	1.12 ± 0.15	6.92 ± 5.39	6.19 ± 4.82
	258	0.64 ± 0.21	13.44 ± 3.21	21.03 ± 5.03	1.14 ± 0.03	5.77 ± 2.51	5.06 ± 2.20
	502	0.87 ± 0.08	3.83 ± 0.98	4.40 ± 1.13	0.76 ± 0.07	5.45 ± 2.59	7.18 ± 3.42

Table 4. Basal and Tetraconazole (Tet)- or Difenoconazole (Dif)-induced *CbCyp51* average expression of three isolates of each C. beticola Haplotypes A-E. Haplotype A isolate 354 has an I387M mutation and is denoted by an asterisk*. All haplotype expression values are averaged from isolate expression values in Table 3**.** Error values represent standard deviation between average expression of each isolate. Basal and Dif- or Tet-induced expression were calculated by Pfaffl method (Pfaffl 2001). X-fold expression values were calculated by dividing induced expression values by basal expression values. Means denoted by a different letter indicate significant differences between haplotypes within a DMI treatment using a one-way ANOVA followed by a Tukey HSD test ($p \leq 0.05$).

Haplotype	Isolate	Expected Tet Response	Basal Exp	Tet-Induced Expression	Tet X-fold Expression	Expected Dif Response	Basal Exp	Dif-Induced Expression	Dif X-fold Expression
A E170_G +L144_G	574	S	0.79 ± 0.19^{a}	10.17 ± 1.70^{a}	13.64 ± 4.58^{a}	S	$0.60 \pm 0.39^{a,b}$	7.14 ± 5.12^{a}	$11.22 \pm 2.29^{a,b}$
	384								
	354*								
B E170_G +L144F_T	150	S	1.37 ± 0.07^{b}	11.17 ± 8.09^{a}	8.11 ± 5.66^{a}	S	1.27 ± 0.11^{c}	5.22 ± 3.72^{a}	3.98 ± 2.70^{b}
	536								
	540								
C E170_G+ L144F_C	222	R	1.25 ± 0.14^{b}	8.82 ± 0.42^{a}	7.15 ± 1.00^{a}	R	$0.43 \pm 0.14^{a,b}$	1.09 ± 0.57^{a}	2.67 ± 1.29^{b}
	493								
	569								
D E170_A +L144_G	227	R	0.61 ± 0.10^{a}	7.52 ± 0.74^{a}	12.73 ± 3.12^{a}	S	0.35 ± 0.03^{b}	4.69 ± 1.75^{a}	13.99 ± 6.19^{a}
	443								
	532								
E E170_A +L144F_C	245	R	0.76 ± 0.12^{a}	6.99 ± 5.59^{a}	10.07 ± 9.49^{a}	R	$1.01 \pm 0.21^{a,c}$	6.04 ± 0.77^{a}	$6.14 \pm 1.06^{a,b}$
	258								
	502								

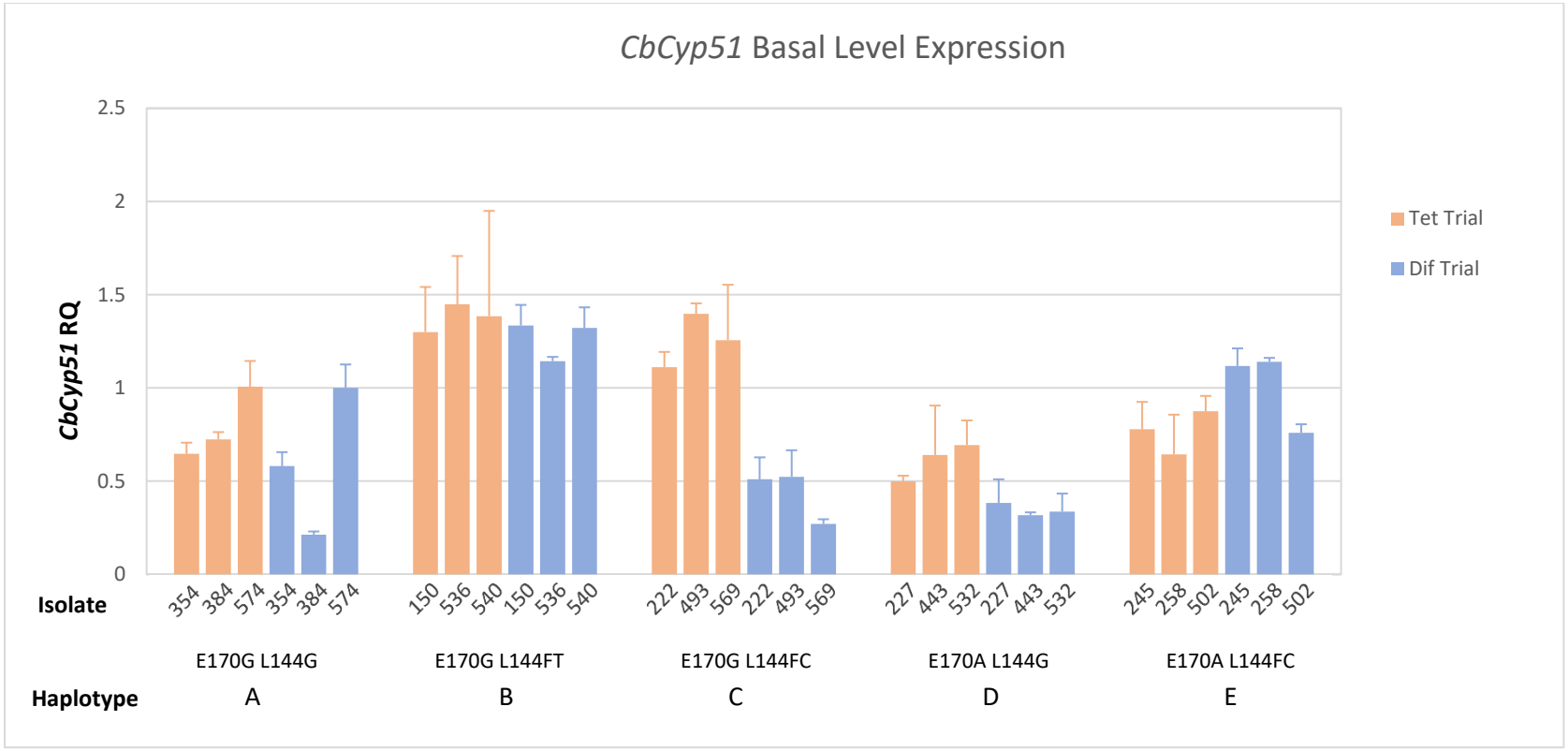


Figure 4. Relative expression of *CbCyp51* across haplotypes without DMI exposure. Values represented by histogram bars indicate the calculated basal expression of *CbCyp51* for each isolate, relative to isolate 574 of each trial. Error bars represent the standard deviation across three biological replicates for each treatment.

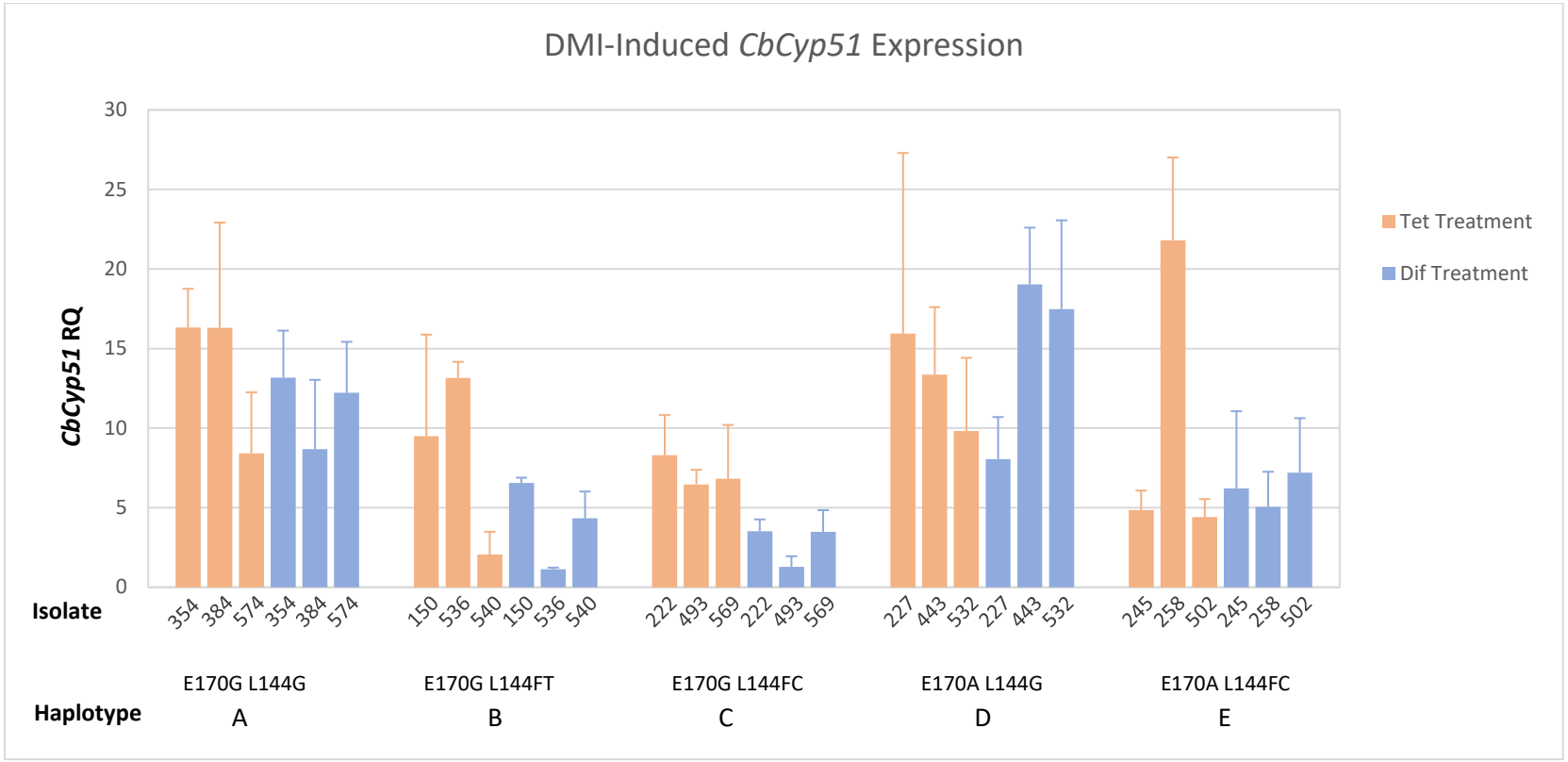
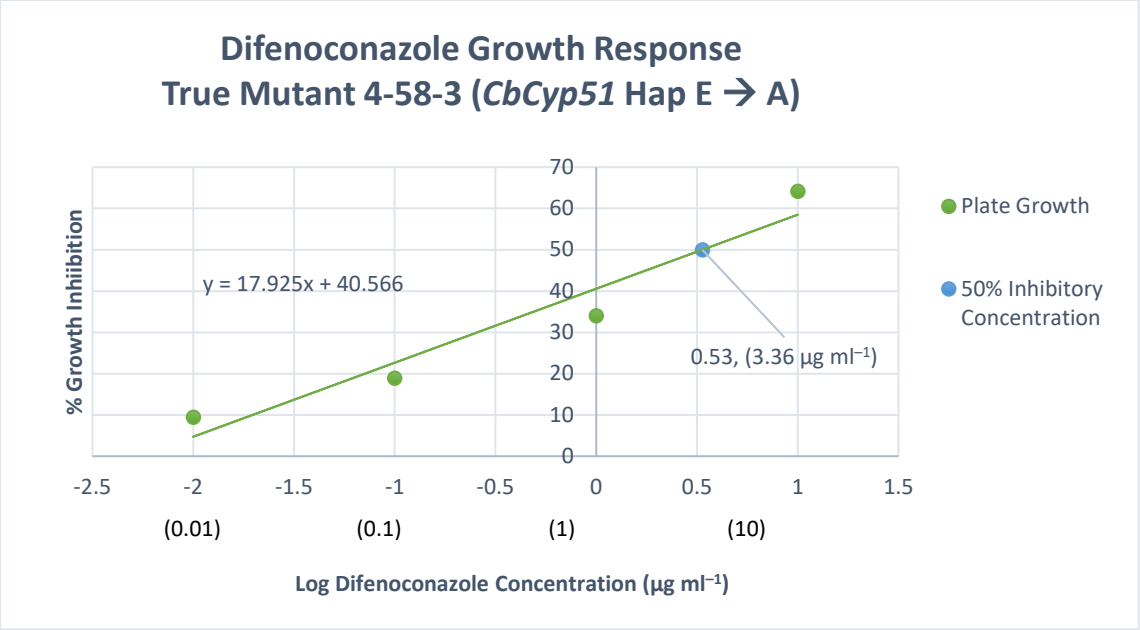


Figure 5. Relative expression of *CbCyp51* across haplotypes after exposure to DMI at 10 µg ml^{-1}. Values represented by histogram bars indicate the calculated X-fold change in expression above the *CbCyp51* basal expression for each isolate. Error bars represent the standard deviation across three biological replicates for each treatment.

2.4.2. Results of DMI resistance assays of *CbCyp51* mutants

To confirm the effects of point mutations in *CbCyp51* on DMI resistance in *C. beticola*, DMI resistance assays were carried out on mutant strains with native *CbCyp51* swapped for different haplotypes. Included with mutant strains were ectopic controls and untransformed controls which were derived from fungal protoplasts and single spored. Most transformants exhibited elevated tetraconazole resistance, regardless of the haplotype change. There were four outlier transformants that showed lowered Tet resistance, which are indicated in the following subsections. The number of *CbCyp51* insertions incurred during the transformation process (*CbCyp51* copy number) was variable between mutants and transformations.

A)



B)

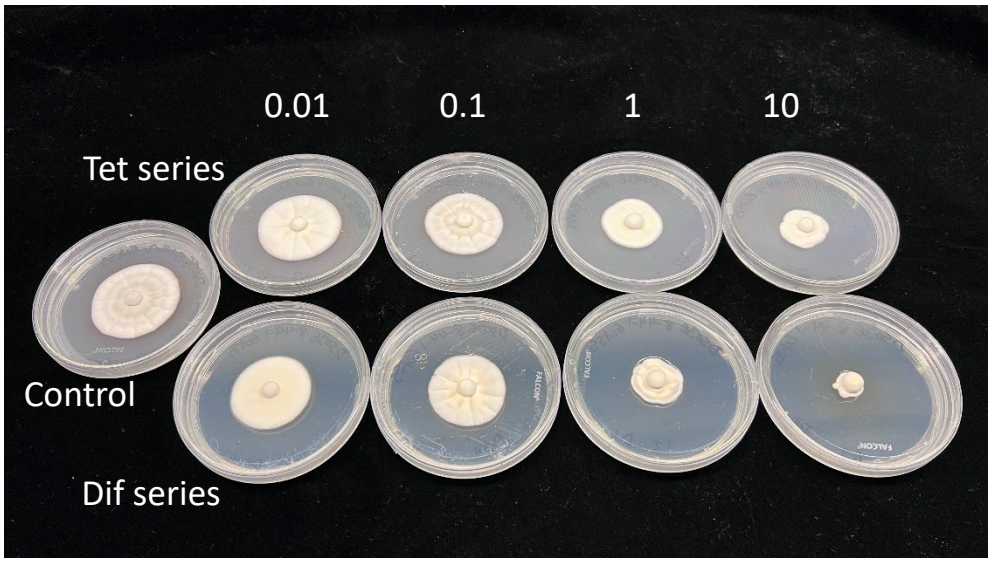


Figure 6. A) Example growth response curve from DMI resistance assay. The blue point indicates the difenoconazole EC_{50} concentration for this biological replicate. B) Example growth pattern on tetraconazole (Tet) and difenoconazole (Dif) resistance assay plates after fifteen days. This biological replicate typifies a resistant response to tetraconazole and a sensitive response to difenoconazole. DMI concentrations are listed in µg ml^{-1}.

2.4.2.1. Haplotype swap E to A

Haplotype E strain 21-611 was mutated to haplotype A. The transformation yielded ten true mutants. All true mutants displayed significantly reduced Dif EC_{50} values relative to wild type, no-DNA controls, and ectopic mutants. Two mutants (4-5 and 4-21) had high *CbCyp51* copy numbers, while all others had a copy number of one. EC_{50} values and *CbCyp51* copy numbers are listed in Table 5.

Table 5. EC_{50} values for mutants of haplotype E strain 21-611 in µg ml^{-1}. Values were calculated using percentage radial growth reduction compared to growth on non-amended media. EC_{50} values were given an upper limit of 100. 'WT' – untransformed background strain. 'Con' – untransformed controls derived from fungal protoplasts. 'Ect' – transformants with unaltered native *CbCyp51*. 'TRUE' – transformants with native *CbCyp51* replacement with *CbCyp51* haplotype A.

Haplotype E Strain 611 → Haplotype A				
Status	Mutant #	Avg Tet EC_{50}	Avg Dif EC_{50}	*CbCyp51* Copy #
WT		22.37	100.00	1
Con	8-5	100.00	100.00	1
Con	8-6	100.00	100.00	1
Ect	4-6	81.05	81.19	1
Ect	4-8	100.00	100.00	1
Ect	4-21	100.00	72.46	2
TRUE	4-1	100.00	1.30	1
TRUE	4-5	81.86	1.26	10
TRUE	4-11	100.00	0.85	1
TRUE	4-19	57.54	1.18	1
TRUE	4-25	100.00	1.77	21
TRUE	4-28	100.00	1.15	1
TRUE	4-34	100.00	1.04	1
TRUE	4-41	100.00	1.51	1
TRUE	4-58	100.00	2.60	1
TRUE	4-64	100.00	1.56	1

Sensitive phenotype Resistant phenotype

2.4.2.2. Haplotype swap E to B

Haplotype E strain 21-611 was changed to haplotype B. The transformation yielded seven true mutants. True mutant 4-17 had two deleted nucleotides immediately 5' upstream of the *CbCyp51* replacement sequence and displayed reduced resistance to both Tet and Dif. Two other true mutants (4-2, 4-18) displayed reduced Dif EC_{50} values. All other true mutants had Dif EC_{50} values comparable to wild type, no-DNA controls, and ectopic mutants. One true and three ectopic mutants had copy numbers higher than one. EC_{50} values and *CbCyp51* copy numbers are listed in Table 6.

Table 6. EC_{50} values for mutants of haplotype E strain 21-611 in µg ml^{-1}. Values were calculated using percentage radial growth reduction compared to growth on non-amended media. EC_{50} values were given an upper limit of 100. 'WT' – untransformed background strain. 'Con' – untransformed controls derived from fungal protoplasts. 'Ect' – transformants with unaltered native *CbCyp51*. 'TRUE' – transformants with native *CbCyp51* replacement with *CbCyp51* haplotype B.

Haplotype E Strain 611 → Haplotype B				
Status	**Mutant #**	**Avg Tet EC_{50}**	**Avg Dif EC_{50}**	***CbCyp51* Copy #**
WT		22.37	100.00	1
Con	8-5	100.00	100.00	1
Con	8-6	100.00	100.00	1
Ect	4-12	100.00	100.00	6
Ect	4-15	61.88	100.00	1
Ect	4-30	93.90	100.00	15
Ect	4-31	32.95	71.85	5
TRUE	4-1	100.00	95.70	1
TRUE	4-2	45.34	3.52	1
TRUE	4-5	100.00	100.00	1
TRUE	4-8	100.00	100.00	1
TRUE	4-17*	8.82	5.48	1
TRUE	4-18	53.56	13.63	1
TRUE	4-20	100.00	100.00	3

Sensitive phenotype Resistant phenotype

2.4.2.3. Haplotype swap D to E, isolate 21-503

Haplotype D strain 21-503 was changed to haplotype E. The transformation yielded four true mutants. Two true mutants (8-26, 8-58) displayed elevated Dif EC_{50} values, while the other two exhibited similar Dif EC_{50} values relative to wild type, no-DNA controls, and ectopic mutants. All true and ectopic mutants had high *CbCyp51* copy numbers. Three transformants displayed reduced tetraconazole resistance (Table 7). EC_{50} values and *CbCyp51* copy numbers are listed in Table 7.

Table 7. EC_{50} values for mutants of haplotype E strain 21-503 in µg ml^{-1}. Values were calculated using percentage radial growth reduction compared to growth on non-amended media.. EC_{50} values were given an upper limit of 100. 'WT' – untransformed background strain. 'Con' – untransformed controls derived from fungal protoplasts. 'Ect' – transformants with unaltered native *CbCyp51*. 'TRUE' – transformants with native *CbCyp51* replacement with *CbCyp51* haplotype E.

Haplotype D Strain 503 → Haplotype E				
Status	Mutant #	Avg Tet EC_{50}	Avg Dif EC_{50}	*CbCyp51* Copy #
WT		13.61	0.50	1
Control	9-1	100.00	2.25	1
Control	9-2	100.00	1.67	1
Ect	8-43	100.00	4.13	12
Ect	8-44	26.00	1.08	7
Ect	8-46	5.99	0.73	18
TRUE	8-7	0.82	1.12	49
TRUE	8-14	0.73	0.62	18
TRUE	8-26	23.57	18.44	42
TRUE	8-58	100.00	18.64	6

Sensitive phenotype Resistant phenotype

2.4.2.4. Haplotype swap D to E, isolate 21-532

Haplotype D strain 21-532 was changed to haplotype E. The transformation yielded four true mutants. All true mutants displayed elevated Dif EC_{50} values relative to wild type, and no-DNA controls. One ectopic mutant (8-48) had a resistant response to Dif (Table 8). True and ectopic mutants had *CbCyp51* copy numbers of one or two. EC_{50} values and *CbCyp51* copy numbers are listed in Table 7.

Table 8. EC_{50} values for mutants of haplotype E strain 21-532 in µg ml^{-1}. Values were calculated using percentage radial growth reduction compared to growth on non-amended media. EC_{50} values were given an upper limit of 100. 'WT' – untransformed background strain. 'Con' – untransformed controls derived from fungal protoplasts. 'Ect' – transformants with unaltered native *CbCyp51*. 'TRUE' – transformants with native *CbCyp51* replacement with *CbCyp51* haplotype E.

Haplotype D Strain 532 → Haplotype E				
Status	**Mutant #**	**Avg Tet EC_{50}**	**Avg Dif EC_{50}**	***CbCyp51* Copy #**
WT		5.92	0.46	1
Control	5-5	100.00	1.69	1
Ect	8-48	100.00	8.32	2
Ect	8-51	100.00	5.04	2
Ect	8-41	100.00	15.05	1
TRUE	8-15	100.00	95.57	2
TRUE	8-24	100.00	100.00	2
TRUE	8-40	100.00	9.78	1
TRUE	8-14	100.00	100.00	1

Sensitive phenotype Resistant phenotype

2.5. Discussion

Different combinations of mutations at the positions of L144 and E170 of cb*Cyp51* have previously been linked to DMI fungicide resistance in *C. beticola*. Confirming the importance of these mutations, and understanding how they contribute to resistance, is necessary for the accurate prediction of resistance levels in field populations. Because differences in *CbCyp51* expression have previously been linked to DMI resistance in *C. beticola* (Bolton et al. 2016), it was questioned whether or not expression of *CbCyp51* may correlate with haplotype. Comparison of *CbCyp51* expression also served as a starting point for investigating the underlying causes of resistance-associated synonymous mutations. Since synonymous mutations do not change the amino acid sequence, it is logical to hypothesize that changes in phenotype linked to such mutations may be caused at the level of transcription or translation. At the level of transcription, differences in coding sequence may influence methylation patterns and chromatin structure (Zhou et al. 2016), as well as recognition by post-transcriptional regulatory elements, which can influence pre-mRNA processing (Plotkin and Kudla 2011). To determine if these factors may underlie observed resistance, we compared expression between haplotypes at the basal level and after exposure to tetraconazole or difenoconazole.

Significant differences in expression were observed between haplotypes in basal level expression for both trials, and in the difenoconazole treatment group. However, the implications of these differences are unclear. The haplotypes which exhibited significantly different basal levels of *CbCyp51* expression were inconsistent between the two trials (Table 4), and the basal expression levels were lower, overall, in the difenoconazole treatment trial (Figure 1). The only discernable difference in experimental design between the control groups of each isolate was the time at which the plugs for starting the liquid culture were taken from the plate. The plugs were taken from each

plate 7 days after growth was initiated for the tetraconazole trial group, and 14 days for the difenoconazole group.

Significant differences were observed between haplotypes D and B, and between haplotypes D and C in *CbCyp51* expression after difenoconazole exposure. Because haplotype D differs at both the L144 and E170 codons relative to haplotypes B and C, there is no conclusion to be drawn at the level of individual mutations. Additionally, because haplotype B predicts sensitivity to both DMIs and haplotype C predicts resistance to both DMIs, there is no trend between resistance and expression to be gathered from these significant differences. No statistically significant differences in expression were observed between haplotypes after exposure to tetraconazole.

In all comparisons, the high degree of variability between isolates within a haplotype, and even between biological replicates of an isolate, creates uncertainty in understanding trends in *CbCyp51* expression. Genetic differences between the isolates chosen may help explain variability in expression level within each haplotype. Variation between biological replicates could be explained by the possible presence of contamination in different samples. Although no strong indication of contamination was observed in any liquid culture, some isolate plates used to start the cultures exhibited fungal contamination following the study. Finally, the reliability of actin as a stable reference gene in *C. beticola* has not been directly evaluated, but rather inferred from literature on RT-qPCR. However, reports on the stability of β-actin expression in various species, cell types, and experimental conditions are mixed (Mori et al. 2008; Dundas and Ling 2012; Panina et al. 2018; Sarwar et al. 2020). It is therefore an assumption that actin expression is stable between isolates and under conditions of DMI exposure.

Future experiments measuring *CbCyp51* expression between haplotypes would benefit from using more isolates to gain a clearer picture of trends within haplotypes. Verification of the stability of reference genes under DMI exposure would help to validate the accuracy of RT-qPCR results. Additionally, adjusting the concentration of DMI added may be more informative than the concentration we used. 10 μg ml^{-1} is a relatively high concentration of both Tet and Dif, and this concentration may totally inhibit the growth of the most sensitive isolates. Exposing the different *CbCyp51* haplotypes to empirically determined concentrations of DMI could reveal transcriptional changes that differ from what we observed.

To confirm the importance of *CbCyp51* mutations in DMI resistance, haplotype swaps were performed through PEG-mediated transformation, and mutants were evaluated for DMI resistance. Mutants were confirmed by sequencing at the genomic location of native *CbCyp51*, and *CbCyp51* copy number was determined with qPCR.

Regardless of which transformation they were derived from, most colonies exhibited elevated tetraconazole resistance, which was also true for the no-DNA controls. This was observed for true mutants, ectopic mutants, and no-DNA controls. Therefore, we believe that the elevated tetraconazole resistance observed is an artifact of the transformation process itself, rather than the result of *CbCyp51* haplotype change. A similar finding was previously reported in *C. beticola*, where gene knockout mutants consistently exhibited elevated tetraconazole resistance regardless of the gene target (Bolton et al. 2016). Despite this finding, some informative changes in difenoconazole resistance were observed in true mutants.

To see if a DMI resistant strain could be made sensitive solely by changing the *CbCyp51* haplotype, a highly resistant haplotype E strain (21-611) was converted to haplotype A. For the haplotype E to A transformation, all ten true mutants exhibited significantly reduced Dif resistance

relative to the wild type, controls, and ectopic mutants. Most mutants had no additional *CbCyp51* insertions, apart from two mutants which had 10 and 21 insertions. Regardless of the number of insertions, these mutants had comparably low Dif EC_{50} values. This result is highly indicative of the importance of *CbCyp51* haplotype in determining resistance to difenoconazole. Because two nucleotides are altered in the transformation from haplotype E (L144F_C+E170_A) to A (L144+E170_G), change in Dif sensitivity based on the individual mutations cannot be directly evaluated. To evaluate these mutations individually, haplotype E could be converted to either haplotype C (L144F_C+E170_G) or haplotype D (L144+E170_A) and tested for DMI resistance.

Haplotype E strain 21-611 was also mutated to haplotype B, the other *CbCyp51* haplotype that predicts sensitivity to all four DMIs. This haplotype swap did not produce a consistent change in resistance to Dif. Only three out of seven true mutants showed significantly reduced Dif EC_{50} values, and only two of these (4-2 and 4-17) were below the resistance threshold. Mutant 4-17 was found through sequencing to be missing two nucleotides 5' upstream of the *CbCyp51* transformation sequence, which was 45 nucleotides upstream of the *CbCyp51* gene and mRNA region. Interestingly, this mutant was also the only transformant from this haplotype swap that showed decreased tetraconazole resistance. A relation between the missing nucleotides and the decreased level of resistance to both DMIs cannot be ruled out. As to why most of the true mutants did not appear to have impacted Dif resistance, a potential explanation is the possibility of linkage between *CbCyp51* haplotype B and other mutation(s) outside of *CbCyp51* that influence resistance. Linked genes have a low rate of recombination between them, so particular mutations in one gene may be commonly inherited with mutations in another (Cutter and Payseur 2013). This can give the appearance of a mutation influencing a phenotypic trait that is actually influenced by linked mutation(s) at a different locus (Cutter and Payseur 2013). In this scenario, other mutations outside

of *CbCyp51* would be the primary cause of DMI sensitivity in haplotype B isolates, so simply swapping the *CbCyp51* haplotype to B would not result in reduced DMI resistance.

To test if difenoconazole resistance could be increased through haplotype swap, haplotype D strain 21-503 was mutated to haplotype E. Surprisingly, this yielded three transformants which showed reduced tetraconazole resistance (ectopic mutant 8-46 and true mutants 8-7 and 8-14). These mutants also displayed low Dif EC_{50} values comparable to the wild-type, control, and the other ectopic mutants. The two true mutants which maintained high Tet resistance also showed elevated resistance to Dif. The mixed results of this transformation may be due in part to the high *CbCyp51* copy numbers seen in all mutants. The fact that as many as 49 copies of the transformation construct had been inserted into the genomes of these mutants could have unpredictable consequences for DMI resistance, or fitness in general.

An additional haplotype D to E swap was made using isolate 21-532. This transformation yielded four true mutants, all of which displayed elevated resistance to Dif. The *CbCyp51* copy number for these mutants was one or two, which may implicate a more accurate representation of the effects of this haplotype swap compared with the 21-503 mutants. However, one ectopic mutant also displayed a difenoconazole-resistant phenotype. Although the EC_{50} value for this ectopic mutant was still significantly lower than values for three out of four true mutants, the result still suggests that the introduction of the *CbCyp51* construct may influence DMI resistance, regardless of genomic location. Considering both haplotype swaps of D to E, a total of 6 out of 8 true mutants displayed notably higher difenoconazole resistance compared to wild-type, no-DNA controls, and most ectopic mutants. These results appear to implicate the influence of *CbCyp51* haplotype in determining difenoconazole resistance. This can be extrapolated to the L144F_C mutation, as this is the only difference between haplotypes D and E.

To consider the effect multiple insertions of *CbCyp51* may have on DMI resistance, copy number was determined with qPCR using a standard curve produced from wild type DNA. While most mutants had a copy number of one or two, all mutants of strain 503 (haplotype D) had much higher than average *CbCyp51* copy numbers. The transformation of this haplotype yielded three mutants which displayed a sensitive response to tetraconazole, making them the only mutants of all four transformations sensitive to Tet. Despite the high copy number seen in these mutants, there appears to be no correlation between copy number and resistance/sensitivity to either Tet or Dif. This at first seems surprising, given reports of *CbCyp51* copy number variation correlating with increased DMI resistance in various fungal pathogens (Chen et al. 2020). However, this may be explained by the unlikelihood that the *CbCyp51* replacement construct used in this study contains the full, functional promoter region, as only 50 base pairs upstream of *CbCyp51* were included. Ectopic insertions of *CbCyp51* would therefore remain untranscribed (unless integrating behind a different promoter), presumably having no effect on *CbCyp51* expression levels. Another notable observation regarding copy number is the existence of multiple ectopic mutants with a copy number of one. Presumably, ectopic mutants have integrated a copy of the transformation construct elsewhere in the genome, which should equate to a copy number of at least two. Their ability to grow on hygromycin B amended media indicates they have a full copy of the hygromycin B resistance gene, but the unaltered native *CbCyp51* sequence indicates the construct has integrated in a different genomic location. A possible explanation for this is that only a partial sequence of *CbCyp51* was integrated along with the selectable marker, which excludes the 212 base pair section of *CbCyp51* amplified for qPCR analysis in this study.

Taken together, the DMI resistance assay results indicate that the mutations constituting the five haplotypes influence DMI resistance, but other factors are likely involved. The haplotype

E to haplotype A swap is intriguing in its consistency, and repeating this swap in another haplotype E strain would indicate whether this is a phenomenon unique to this strain, or if it is a trend that can be extrapolated to other isolates. However, the question remains why a swap to haplotype B, which also predicts sensitivity, had usually no effect on Dif resistance when using the same strain. Additionally, while the haplotype D to E swap produced elevated resistance in two different isolates, these results were also mixed. Performing additional haplotype swaps may be informative for understanding the effects of these mutations, but mixed results and artificially elevated tetraconazole levels present obstacles. One potential solution to understanding effects of haplotype swap on tetraconazole resistance would be to instead measure prothioconazole sensitivity, as these two fungicides show strong cross-resistance behavior (Rangel et al. unpublished data). Another solution would be to perform transformations using methods that do not involve the generation of protoplasts, such as agrobacteria-mediated transformation.

Future goals for this project will involve the generation and study of additional *CbCyp51* haplotype-swap mutants. In addition to DMI resistance assays, metrics indicating levels of transcription and translation can be compared between mutant and wild type strains. *CbCyp51* expression analysis on mutant versus wild type strains would eliminate potential background effects incurred from genetic differences between isolates within a haplotype. This way, expression changes based solely on the presence of *CbCyp51* mutations could be evaluated. Effects of *CbCyp51* mutations, especially synonymous, can also be evaluated at the level of translation. Quantifying levels of ergosterol and comparing between mutants or wild type strains from different haplotypes could reveal whether or not the efficiency of the ergosterol biosynthesis pathway is impacted by changes in *CbCyp51*. Ribosome profiling, which quantifies the RNA sequences bound by ribosomes at the time of tissue harvest, is a plausible way to directly measure translation

(Ingolia et al. 2012). All of these methods could be utilized on true mutants, ectopics, no-DNA controls, and wild types of various haplotypes, with and without exposure to various levels of different DMI fungicides. Beyond elucidating the role these mutations play in fungicide resistance, studying their effects on general fitness in planta could be informative for predicting the likelihood of the retention of these mutations in the absence of DMI pressure in the field. Greenhouse or field inoculations could also be used to test the resistance response in isolates of different haplotypes under conditions approximating a typical growing season. The potential studies involving the five primary RRV *CbCyp51* haplotypes of *C. beticola* can serve to increase our understanding of DMI resistance, which will improve the ability of researchers to inform sugar beet growers on how to manage CLS in an accurate and timely manner.

2.6. Conclusion

To conclude, *CbCyp51* expression was measured between the five common *CbCyp51* haplotypes of the Red River Valley with and without exposure to DMIs. Significant differences were observed between haplotypes at the basal level, and after exposure to difenoconazole, but these differences were not informative. Testing additional isolates, under different concentrations, and potentially with an alternate reference gene, may yield different results. The native *CbCyp51* haplotype was successfully changed in three different isolates through PEG-mediated transformation, and DMI resistance assays were used to determine EC_{50} values for tetraconazole and difenoconazole. Elevated tetraconazole resistance was observed for most transformants, regardless of native *CbCyp51* sequence. Consistent, significant reduction in difenoconazole resistance was observed for true mutants of the haplotype change from E to A. Difenoconazole resistance was mostly unchanged in mutants of the haplotype change from E to B. Most mutants of the haplotype D to E change exhibited higher difenoconazole resistance. The DMI resistance

assays of haplotype-swap mutants indicate that *CbCyp51* SNPs influence DMI resistance in *C. beticola*, supporting previous research on the identification of these resistance-associated mutations. Future studies will involve additional haplotype swaps, and evaluation of mutants for transcriptional and translational differences to uncover the mechanisms behind *CbCyp51* mutation-driven DMI resistance.

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