Investigation of Cytochrome P450 Monooxygenases in S. homoeocarpa for Chlorothalonil Biotransformation

INVESTIGATION OF CYTOCHROME P450 MONOOXYGENASES IN *S. HOMOEOCARPA* FOR CHLOROTHALONIL BIOTRANSFORMATION

A Thesis Presented

By

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ABSTRACT

INVESTIGATION OF CYTOCHROME P450 MONOOXGENASES IN *S. HOMOEOCARPA* FOR CHLOROTHALONIL BIOTRANSFORMATION

MAY 2017

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Sclerotinia homoeocarpa (F.T. Bennett) is one of the most economically important pathogens on high amenity cool-season turfgrasses where it causes dollar spot. Due to decades of over-reliance and repeated chemical treatments, *S. homoeocarpa* has developed resistance and insensitivity to multiple classes of fungicides. To understand the genetic mechanisms of fungicide resistance, the whole genomes of two strains with varying resistance levels to fungicides, were sequenced. In unpublished data (Sang et al.), a RNA-sequencing analysis revealed three CYP450s that were validated to play a functional role in *S. homoeocarpa'*s resistance against different fungicide classes. We also identified CYP450 metabolic action on the multi-site mode of action fungicide chlorothalonil. Chlorothalonil is an extensively used contact fungicide and has been known to be persistent in soils. Yet, *S. homoeocarpa* resistance to chlorothalonil has not been reported in the field. High Performance Liquid Chromatography (HPLC) indicated faster rates of chlorothalonil biotransformation by CYP450 overexpression strains when compared to the wild-type. We show by GC-MS that the primary transformation intermediate found in soils, 4-hydroxy-2,5,6 trichloroisophthalonitrile is produced by CYP450s' metabolism.

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CHAPTER 1

INTRODUCTION

Sclerotinia homoeocarpa (F.T. Bennett) is one of the most economically important pathogens on high amenity turfgrass (golf courses, athletic fields and home lawns) and causes the turfgrass disease, dollar spot. This disease affects both the quality and appearance of turfgrass and persists from late spring to autumn. *Sclerotinia homoeocarpa* causes foliar lesions, which can progress to small bleached patches (3-6 cm in diameter) of turf that negatively affect key traits such as ball roll (Smiley et al., 2005). Fungicides are the most effective way of controlling dollar spot, but repeated use has led to the development of *S. homoeocarpa* strains resistant to many fungicide classes (Popko et al., 2012; Allan-Perkins et al., 2017). There is a strong need to understand the genetic mechanisms of resistance for fast and accurate diagnosis, development of new drug targets, and ultimately to develop integrated pest management strategies to minimize inefficient fungicide use.

Many species of pathogenic fungi have gained insensitivity or resistance to fungicides due to their overreliance and misuse in clinical and field applications. In 2012, Popko et al. assessed the practical field resistance of populations of *S. homoeocarpa* to the Demethylation Inhibitor (DMI) class of fungicides. One of these populations showed a bimodal population with almost half of the isolates being highly sensitive to DMIs and the other half being highly resistant. Two isolates from this population were chosen as models to explore the mechanisms of DMI insensitivity using molecular and genomic

tools. The first isolate was HRS10, which is sensitive to DMI fungicides and the second was HRI11, which is insensitive to DMI fungicides.

The biochemical mode of action of DMI fungicides is to block the production of ergosterol, a component of cell membrane biosynthesis in fungi. More specifically, DMI fungicides target the *CYP51* gene that codes for the enzyme sterol 14 α -demethylase. A direct mutation in the *CYP51* gene often confers fungicide insensitivity (Ma and Tredway, 2013). Hulvey et al. (2012) reported minor levels of *CYP51* overexpression in DMI insensitive isolates including HRI11. Additionally, fungi may use indirect mechanisms, such as removing xenobiotics through ATP Binding Cassette (ABC) transporter proteins to prevent fungicide concentrations from reaching harmful levels within the cell (Paul et al., 2014). These indirect mechanisms may play a larger role in resistance than direct mutations in *CYP51*. Many *Aspergillus fumigatus* isolates have shown azole fungicide resistance that is not related to a direct mutation in *CYP51* (Bueid et al., 2010). Hulvey et al. (2012) observed higher levels of *ShatrD* (an ABC transporter gene) expression in DMI insensitive isolates (constitutive and induced, 1 hour post exposure to a DMI fungicide) compared to DMI sensitive isolates.

The isolate HRI11 exhibits cross resistance to DMIs and plant growth regulators, and multidrug resistance (MDR) to propiconazole (DMI), succinate dehydrogenase inhibitors (SDHI), methyl benzimidazole carbamates (MBC), dicarboximide fungicide classes, and plant growth regulators (Bishop et al., 2008; Putnam et al., 2010; Allan-Perkins et al., 2017). RNA-Sequencing analysis of HRI11 revealed higher expression of proteins linked to fungicide detoxification (Cytochrome P450 monooxygenases, conjugases, and ABC transporters) (unpublished data, Sang et al.). Quantitative-Real-

Time-PCR (qRT-PCR) showed that two ABC transporters, *ShatrD* and *ShPDR1*, and one CYP450 monooxygenase (unpublished data, Sang et al.) were overexpressed in multidrug resistant field isolates (Sang et al., 2015).

Sang et al. (2015) reported overexpression of an ABC transporter *ShPDR1* in isolate HRI11 both constitutively and in the presence of DMI, dicarboximide and SDHI fungicides and further corroborated these findings with yeast complementation assays. It was found that ShPDR1 overexpression results in an MDR phenotype to chemically different fungicide classes. Recently, an updated RNA-sequencing analysis identified the overexpression of additional Phase I cytochrome P450 monooxygenases in the MDR isolate of *S. homoeocarpa.* A total of three CYP450 genes were found to be associated with Phase I metabolizing enzymes of a xenobiotic detoxification system in filamentous ascomycete fungi. Further work showed both Phase I CYP450 monooxygenases and Phase III ABC transporters are regulated by a Xenobiotic Detoxification Regulator (ShXDR1), and MDR strains harbor a gain of function dominant mutation in XDR1 (Sang et al., 2017).

In every organism, CYP450s play essential and specialized roles in primary and secondary metabolism. In ascomycete fungi, CYP450s are hugely diverse and many are involved in specialized processes that allow fungi to occupy specific niches (Chen et al., 2014; Deng et al., 2007). Ascomycetes utilize CYP53 P450s to specifically detoxify benzoate, a common antifungal produced by plants. However, in basidiomycetes, CYP53 P450s are also involved in the degradation of wood (Jawallapersand et al., 2014). *Sclerotinia homoeocarpa*'s genome encodes for three CYP450s identified as CYP561, CYP65, and CYP68. Overexpression strains of CYP561, CYP65 and CYP68 exhibited

resistance to propiconazole and flurprimidol, but showed differing sensitivities to boscalid and iprodione (unpublished, Sang et al.). We believe that similar to CYP53, the three CYP450s are substrate specific to different fungicide classes, and work in tandem to form a robust Phase I detoxification system. In chemical insensitivity assays (unpublished), the overexpression strains displayed varying insensitivities to the multisite fungicide chlorothalonil. Chlorothalonil resistance has been reported in isolates of the plant pathogen *Botrytis cinerea* (Zhang et al., 2009), but no field resistance has been reported in *S. homoeocarpa*. In addition, the mechanism of *B. cinerea*'s resistance to chlorothalonil is currently unknown. Furthermore, one of the known primary degradates of chlorothalonil in soils is 4-hydroxy-2,5,6-trichloroisophthalonitrile, which has been shown to be 30 times more toxic to mice than chlorothalonil, as well as more mobile and persistent in soil (Cox, 1997). We hypothesized that the 4-hydroxy degradate of chlorothalonil is a product of xenobiotic detoxification through CYP450 metabolism.

Sclerotinia homoeocarpa exhibits multiple mechanisms that confer multidrug resistance. However, it is likely that there are additional and redundant MDR mechanisms not yet discovered. For example, our lab has identified the gene in *S. homoeocarpa* which encodes a homolog of PDR1 found in *S. cerevisiae*, but we have not yet identified a homolog of PDR3, which is also known to confer MDR in *S. cerevisiae* (Schüller et al., 2007). Additionally, there are a number of other transcription factors that are known to confer resistance in other fungal species. In *C. albicans*, Tac1 is responsible for the up-regulation of ABC transporters CDR1 and CDR2 in azole-resistant isolates (Coste et al., 2004). Also in *C. albicans*, UPC2 binds to the promoters of genes that code for ergosterol biosynthetic and sterol uptake proteins (Yang, 2015). A yet to be characterized homolog

of this transcription factor may be responsible for overexpressing the gene target in *S. homoeocarpa* (*CYP51*). Currently, it is unknown whether or not HRI11 utilizes these additional mechanisms to gain resistance to fungicides.

Mechanisms of resistance found in *S. homoeocarpa* are possibly conserved mechanisms to be investigated in other fungal species. *Candida albicans* is the most prevalent human fungal pathogen, found particularly within individuals with compromised immune systems (Selmecki et al., 2006). *Candida albicans* has developed insensitivity to azole fungicides utilizing similar mechanisms as those found in *S. homoeocarpa*. *B. cinerea* is an economically important plant pathogen that infects vegetables and fruits worldwide that also displays multidrug resistant phenotypes (Kretschmer et al., 2009). While there are similarities between the major gene families involved in xenobiotic detoxification in human pathogenic and plant pathogenic fungi, the evolution of these mechanisms can be different between species, or even isolates. Novel mechanisms are potential drug targets, especially if the mechanism is prevalent in other fungal pathogens. Identifying new mechanisms is also important for development of a fast and accurate diagnostic tool for resistance detection in the field.

CHAPTER 2

DRAFT GENOME SEQUENCES OF THE TURFGRASS PATHOGEN *SCLEROTINIA HOMOEOCARPA* (HRS10 AND HRI11)

Published work: Green, R., Sang, H., Chang, T., Allan-Perkins, E., Petit, E., & Jung, G. (2016). Draft Genome Sequences of the Turfgrass Pathogen *Sclerotinia homoeocarpa*. Genome Announcements, 4(1), e01715–15.

Abstract

Sclerotinia homoeocarpa (F.T. Bennett) is one of the most economically important pathogens on high amenity cool-season turfgrasses where it causes dollar spot. To understand the genetic mechanisms of fungicide resistance, which has become highly prevalent, the whole genomes of two strains with varying resistance levels to fungicides, were sequenced.

Genome Announcement

Sclerotinia homoeocarpa, the causal agent of dollar spot disease, is the most economically important cool season turfgrass pathogen. This sterile ascomycete fungi has developed cross resistance and multiple resistance to the demethylation inhibitor (DMI), methyl benzimidazole carbamates (MBC), and dicarboximide fungicide classes (Detweiler et al., 1983, Bishop et al., 2008, Koch et al., 2009, Putnam et al., 2010). Two isolates of *S. homoeocarpa,* HRS10 and HRI11, were collected from individual symptomatic leaf blades of creeping bentgrass (*Agrostis stolonifera*) in 2009 (Popko et al., 2012). HRI11 displays insensitivity to DMI fungicides, as well as other chemically

unrelated fungicide classes, while HRS10 is sensitive to all fungicide classes (Hulvey et al., 2012; Sang et al., 2015).

The isolates were preserved on oat seeds (*Avena sativa* L.) according to methods described previously (Chakraborty et al., 2006). Both isolates were transferred to potato dextrose agar (Difco Laboratories, Detroit, MI) and then sub-cultured after four days onto potato dextrose broth and grown for an additional four days. Mycelia were freeze-dried using a lyophilizer (Labconco, Kansas City, MO), and total genomic DNA was extracted using a modified cetyltrimethylammonium bromide method (Cubero et al., 1999).

Libraries were made using a combination of PacBio CLR reads with 20k inserts at HTSF (North Carolina University, USA) and 2x100 Illumina HiSeq reads (Macrogen, Seoul, Korea). Over 38,000,000 Illumina HiSeq and 1,420,000 Pacbio CLR reads were generated for each isolate. The Illumina reads were polished using default parameters with Trimmomatic (Bolger et al., 2014). *De novo* assembly was performed using SPAdes Version 3.6.1 (Bankevich et al., 2012) with k-mer lengths of 33, 55 and 77. Contigs greater than 5,000 base pairs and PacBio CLR reads were used for scaffolding with SSPACE-LongRead (Boetzer & Pirovano, 2014). Scaffolding upgraded the HRS10 draft genome to 258 scaffolds and a total length of 43,384,370 bp (N_{50} 709,078), with a largest scaffold of 1,993,158 bp and a G+C content of 43.83%. The HRI11 assembly was upgraded to 232 scaffolds and a total length of 42,272,096 bp (N_{50} 600,417) with a largest scaffold of 1,672,908 bp and a G+C content of 43.36%.

Repeats were masked using RepeatMasker and the fungal repetitive elements database from RepBase (Jurka et al., 2005). 1,272,222 base pairs were masked in HRS10 (3.01%), and 1,353,753 base pairs were masked in HRI11 (3.12%). Using the masked

genomes, 12,216 and 12,912 proteins were annotated for HRS10 and HRI11, respectively, using RNA transcripts from *Sclerotinia homoeocarpa* (Hulvey et al., 2012) as cDNA hints with Augustus (Stanke & Morgenstern, 2005).

Previous reports (Hulvey et al., 2012; Sang et al., 2015) have revealed multiple mechanisms of fungicide resistance conferred in isolates HRS10 and HRI11. ATP-binding cassettes (ABC) transporters have been shown to be up-regulated in the absence and presence of fungicide, and contribute to insensitivity to multiple fungicide classes. Moreover, zinc-finger proteins and other transcription factors play a large role in *S. homoeocarpa*'s detoxification of xenobiotic substances (Sang et al., 2015). These transcription factors and their DNA-binding domains can now be searched on a genome-wide level for comparison between HRS10 and HRI11.

Nucleotide Sequence Accession Numbers

This Whole Genome Shotgun project has been deposited in GenBank under the accession no. LNGN00000000 for HRS10 and no. LNKV00000000 for HRI11. The versions described in this paper are the first versions, LNGN01000000 and LNKV01000000, respectively.

CHAPTER 3

CHLOROTHALONIL BIOTRANSFORMATION BY CYTOCHROME P450 MONOOXYGENASES IN *SCLEROTINIA HOMOEOCARPA*

Abstract

Cytochrome P450s have been shown to play a vital role in the xenobiotic detoxification system of *Sclerotinia homoeocarpa,* the causal agent of the turfgrass disease dollar spot. In unpublished data (Sang et al.), three CYP450s were validated to play a functional role in resistance against different fungicide classes including propiconazole and plant growth regulator, flurprimidol. The three CYP450s were identified as belonging to the CYP561, CYP65, and CYP68 families, which were found to share limited homologs when compared to the "CYPomes" in *S. homoeocarpa* and two related plant pathogenic fungal species (*Sclerotinia sclerotiorum* and *Botrytis cinerea*). The active sites were compared by protein fold prediction and cavity topology analysis to gain insight on the CYP450s' substrate-specificity. We also identified CYP450 metabolic action on the multi-site mode of action fungicide chlorothalonil. Chlorothalonil is an extensively used contact fungicide and has been known to be persistent in soils. Yet, *S. homoeocarpa* resistance to chlorothalonil has not been reported in the field. High Performance Liquid Chromatography (HPLC) indicated faster rates of chlorothalonil biotransformation by the CYP561 and CYP65 overexpression strains when compared to the wild-type and overexpression strain CYP68. We also show by GC-MS that the primary transformation intermediate found in soils, 4-hydroxy-2,5,6 trichloro-isophthalonitrile is produced by CYP450s' metabolism.

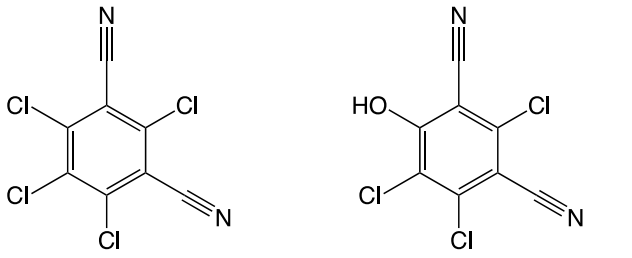
Introduction

Cytochrome P450 monooxygenases (CYP450s) are common enzymes found in every living kingdom. CYP450s play a vital role in the primary and secondary metabolism and catalyze multiple physiological reactions. In ascomycete fungi, CYP450s are hugely diverse and many are involved in specialized processes that allow fungi to occupy specific niches (Chen et al., 2014; Deng et al., 2007). Because of their potential to degrade xenobiotics, fungal CYP450s are of great interest in biotechnology and bioremediation industries. For example, the white rot fungus *Phanerochaete chrysosporium* is of specific interest because it utilizes CYP450s to degrade a multitude of xenobiotic compounds (Syed & Yadav, 2012). In addition, CYP63A2 and CYP5136A3 from *P. chyrsosporium*, can degrade recalcitrant polycyclic aromatic hydrocarbons (Durairaj et al., 2016).

Sclerotinia homoeocarpa, the causal agent of dollar spot disease, is the most economically important cool-season turfgrass pathogen. This sterile ascomycete fungus has developed cross-resistance and multiple resistance to the demethylation inhibitor (DMI), methyl benzimidazole carbamates (MBC), and dicarboximide fungicide classes, and plant growth regulators (Bishop et al., 2008; Putnam et al., 2010; Allan-Perkins et al., 2017). Recently, our lab has discovered and validated through RNA-seq and molecular genetics approaches that three CYP450s are involved in resistance to multiple fungicide classes mediated by xenobiotic detoxification (Sang et al., unpublished). Strains overexpressing each CYP450 were generated to study their metabolizing function in xenobiotic detoxification. CYP450 overexpressing strains were named by their respective

CYP450 family and identified as CYP561, CYP65, and CYP68. Initial fungicide sensitivity screenings suggested the overexpression strains were more insensitive to chlorothalonil than the wild-type strain.

Chlorothalonil (2,4,5,6-tetrychloroisophthalonitrile) (Fig. 1) is a broad-spectrum, non-systemic, contact fungicide commonly used for preventative control of foliar diseases of commercial crops and turfgrass (Hladik & Kuivila, 2008; Allan-Perkins et al., 2017). Repeated sprays of chlorothalonil are subject to runoff and their presence in surface and groundwater has been of concern. Furthermore, chlorothalonil has been reported to photodegrade into several compounds in water and soil (Kwon & Armbrust, 2006). The predominant microbial biotransformation product is 4-hydroxy-2,5,6-trichloroisophthalonitrile (Fig. 1), which has been shown to be 30 times more toxic to mice than chlorothalonil, as well as more mobile and persistent in soil (Cox, 1997).



Chlorothalonil

4-hydroxy-2,5,6-trichloroisophthalonitrile

Figure 1. Chemical structures of chlorothalonil and 4-hydroxy-2,5,6-trichloroisophthalonitrile.

Currently, only two microbial degradation pathways for chlorothalonil have been identified. A glutathione S-transferase in *Ochrobactrum anthropic* SH35B was able to act on the chlorine atoms of chlorothalonil, and *Pseudomonas* sp. CTN-3 utilizes a hydrolytic

dehalogenase to substitute a hydroxyl on the 4-chlorine atom (Wang et al., 2010). The present study is the first report of a CYP450 directly modifying chlorothalonil.

This study shows CYP450 enzymes involved in Phase I of a detoxification system in *S. homoeocarpa* that are able to biotransform chlorothalonil. We hypothesized that the three CYP450s are phylogenetically unique, and that their large active-site cavity plays a role in their ability to detoxify chemically unrelated fungicide classes. Our HPLC results suggest that CYP561 has the highest rate of chlorothalonil biotransformation over CYP65 and CYP68. We also show that 4-hydroxy-2,5,6-trichloroisophthalonitrile was formed in cultures incubated with chlorothalonil, which is the primary transformation intermediate found in chlorothalonil treated soils (Van Scoy & Tjeerderma, 2014).

Materials and Methods

Mining and phylogenetic comparisons of CYP450s in *S. homoeocarpa, B. cinerea, and S. sclerotiorum*

All of the annotated CYP450s in *S. homoeocarpa* and two related species: *B. cinerea* and *S. sclerotiorum* were identified by HMMER 3.0 (http://hmmer.org), searching with hidden Markov Models of the Pfam seed alignment flatfile of PF00067. A total of 96, 126, and 91 CYP450s were mined from the publically accessible genomes *S. homoeocarpa, B. cinerea, and S. sclerotiorum,* respectively (Green et al., 2016; Amselem et al., 2011). Families were assigned to each CYP450 by methods described in Chen et al. (2014). Briefly, BLASTP comparisons against the CYP450 database resulting in similarity to the top hit over 40% were assigned.

Structure-based alignment, protein homology and modeling

We used the PHYRE2 server to generate 3D protein models of the three CYP450s (Kelley et al., 2015). Protein models from PHYRE2 were structurally aligned using the online tool PDBeFold (Krissinel & Henrick, 2004). The same protein models were analyzed by CASTp, using the default 1.4 angstrom probe, to survey the topology of their active site cavities (Dundas et al., 2006). The crystal structure of CYP51 from *Saccharomyces cerevisiae* (PDB code 5UL0) was used as a comparison for active site cavity size (Monk et al., unpublished).

In vitro sensitivity assay

HRS10 and the CYP450 overexpression strains were transferred to minimal media with or without 1 ppm of chlorothalonil (Daconil Ultrex 82.5WG, Syngenta Crop Protection). Mycelial growth was measured after four days growth and relative mycelial growth (RMG) was calculated for each isolate. CYP561 was transferred to potato dextrose agar (PDA), due to its extremely slow growth on minimal media, and RMG was calculated as previously described after two days growth. RMG data was analyzed separately based on the growth media used. The two groups (PDA and minimal media) were subject to an analysis of variance and means were separated using Fisher's Protected LSD ($P = 0.05$).

HPLC analysis

Potato dextrose broth (25 mL) with and without mycelia (approximately 1 g) of strain HRS10 and CYP561, CYP65, and CYP68 overexpressed strains were supplemented with chlorothalonil (10 μg ml^{-1}, Sigma, 99%) and cultured at 25 °C (100 rpm) in biological triplicates. The sample was prepared at each time point (0, 12, 24, 36, 48, and 72 h) by a methanol extraction method (Im et al., 2016). Biomass was weighed before and after the 72 hours, but no significant growth was observed in any of the replicates. An Agilent 1200 Series HPLC equipped with a diode array detector (DAD) was used for the detection and quantification of chlorothalonil. Separation was performed on an Agilent Eclipse XDB C18 column (4.6 mm × 150 mm, 5 micron) using a 12-min linear gradient of I in water (50% to 90%) at a flow rate of 1.5 ml min^{-1}. The DAD was

set at 254 nm to provide the real-time chromatogram, and the UV/Vis spectra from 190 to 400 nm were recorded for detection of transformation products.

GC-MS Analysis

A reference standard of 4-hydroxy-2,5,6-trichloroisophthalonitrile was purchased from Sigma (99%). Mycelia (2 g) of HRS10 was supplemented with 10 μg ml^{-1} of chlorothalonil and cultured at 25 °C (100 rpm). After 7 days, mycelium was removed and the analytes were extracted through SAX/PSA cartridges using acetonitrile/toluene (75:25 v/v), as described by Duca et al., 2014. The analytes were derivatized to trimethyl silyl derivatives by BSTFA:TMCS (99:1 v/v) in a 1:1 ratio, and heated to 70 °C for 1h, as described by Hladik & Kuivila (2008). Injections were made into an Agilent 6890 GC with 5973 MS detector. The column was a J&W DB-5ms column with 30m x 250 um, 0.25 um film thickness. 1 μL sample injections used a 10:1 split, and He carrier gas flow was set to 1 $mLmin^{-1}$. The injector and MS source temperatures were held at 300 °C and 250 °C, respectively. The oven was held at 90 °C , held for 3 minutes, then ramped up to 90-185 °C at 10 °C $minute^{-1}$ with a 4 minute hold time, followed by ramp up to 185-300 °C at 20 °C/$minute^{-1}$ with 2 minute final hold time. Mass spectra were required in EI mode.

Results

Phylogenetic and protein homology comparisons of CYP561, CYP65, and CYP68

A total of 96, 126, and 91 CYP450s were mined from the genomes *S. homoeocarpa, B. cinerea,* and *S. sclerotiorum,* respectively (Green et al., 2016; Amselem et al., 2011). The assignment of families resulted in 13 clades comprising of 59, 77, and 50 assigned CYP450s from *S. homoeocarpa, B. cinerea, and S. sclerotiorum,* respectively (Fig. 2).

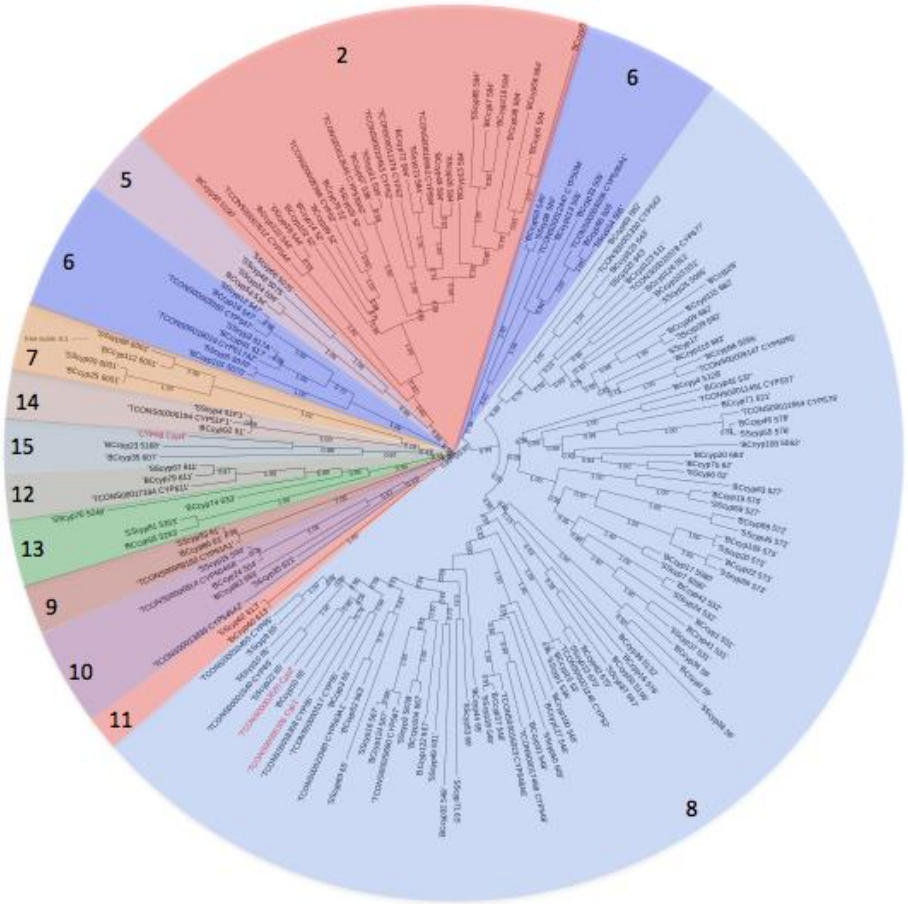


Figure 2. Phylogenetic tree of the annotated CYP450s from *S. homoeocarpa, B. cinerea,* and *S. sclerotiorum.* Each color corresponds to the 12 clades that were found to be present in these ascomycete fungi. *S. homoeocarpa* CYP561 (Clade 15), CYP65 (Clade 8), and CYP68 (Clade 8) are shown in red.

The phylogenetic tree of the annotated CYP450s resulted in identifying few closely related homologs for those three CYP450s investigated in this study. *S. sclerotiorum* or *B. cinerea* did not contain any CYP450s from the CYP561 or CYP68 families (Table 1). The CYP65 family was the largest in *S. homoeocarpa* (8), compared to the other fungi tested. The lack of similar family members or highly similar homologs among the related fungi hints that these ShCYP561, ShCYP65, and ShCYP68 may be specific for involvement in the process of xenobiotic detoxification in *S. homoeocarpa*.

Table 1. Comparison of numbers of three CYP450 families among *S. homoeocarpa, S. sclerotiorum* and *B. cinerea.*

CYP450 family	Number of family members in annotated proteomes		
	S. homoeocarpa	*S. sclerotiorum*	*B. cinerea*
CYP561	1	0	0
CYP65	8	6	3
CYP68	2	0	0

Structure based alignment revealed sequence identities of 33.9% between ShCYP561 and ShCYP65 (RMSD: 1.682), 14.2% between ShCYP65 and ShCYP68 (RMSD: 2.33), and 12.2% between ShCYP561 and ShCYP68 (RMSD: 2.462). The shared RMSD and Q-score for three ShCYP450s was 2.185 and 0.4194, respectively. Sequence alignment did not reveal any highly conserved regions shared between the ShCYP450s, however there was a surprising amount of similarity in the secondary structure shown in Fig. 3.

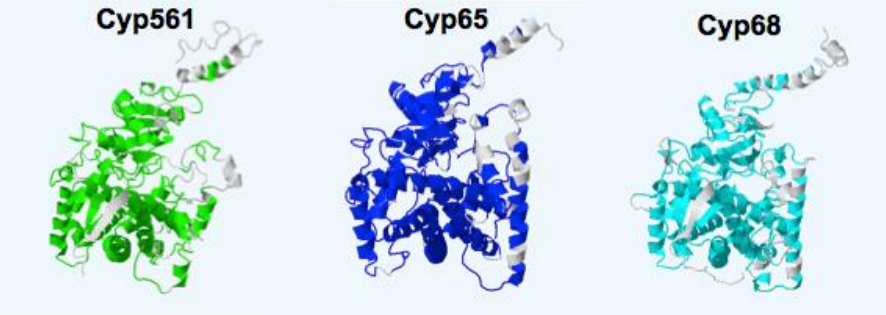


Figure 3. Protein homology comparisons of ShCYP561, ShCYP65 and ShCYP68. The colored regions indicate similar secondary structure, while the regions shown in gray indicate dissimilar regions.

We also compared the sizes of the cavities and substrate access channels among the three ShCYP450s and closest ortholog based on the phylogenic tree (Table 2). Consistently, the xenobiotic detoxifying ShCYP450s have an almost 2-fold increase in respective areas when compared to their closest homolog. In addition to area, volume size is greatly increased, except in the case for ShCYP65. A larger active site cavity size may be an important aspect for CYP450s in ascomycete fungi involved in xenobiotic detoxification.

Table 2. Comparative analysis of active sites of three functionally characteristic CYP450s in *S. homoeocarpa.*

CYP450 families	Length*	Area ($Å^2$)	Volume ($Å^3$)
CYP561	514 (513[a])	4293 (2741)	6007 (3572)
CYP65	480 (347)	3524 (1885)	4843 (5270)
CYP68	511 (433)	4850 (3211)	6969 (5386)
CYP51a1	528	1746	2479

* Length of protein is represented by number of amino acids of the whole protein.
$Å^2$ Area of largest cavity in the CYP450 protein, predicted from pdb files from PHYRE2 protein fold prediction.
$Å^3$ Volume of largest cavity in the CYP450 protein.
[a] Value in parentheses corresponds to the closest related homolog based on the phylogenetic tree.

***In vitro* sensitivity assays**

In vitro sensitivity assays were conduced to further elucidate classes of fungicides and antifungals that are targets of the Phase I involved CYP450s. The CYP450 overexpressing strains displayed varying insensitivities to the fungicide chlorothalonil at 1ppm (Table 3). ShCYP561 was grown on nutrient PDA instead of minimal media due to very slow growth. ShCYP65 and ShCYP68 showed significant insensitivity to chlorothalonil when compared to the wild type. ShCYP561 and the wild-type were not statistically significant. The active compound in the fungicide commercial product Daconil is chlorothalonil, and thus further experiments were carried out using chlorothalonil.

Table 3. Relative mycelial growth (%) of CYP450 overexpression strains

Strains	MM[a] RMG %	PDA[b] RMG %
HRS10	41.8 b[c]	
ShCyp65	57.5 a	
ShCyp68	60.5 a	
HRS10		26.0
ShCYP561		30.0
P value	0.0093	0.1560

[a] Isolates grown on minimal media for four days.
[b] Isolates grown on PDA for two days.
[c] Mean separation was conducted using Fisher's Protected LSD ($P = 0.05$).

Biotransformation rate of chlorothalonil by ShCYP561, ShCYP65, and ShCYP68 overexpression strains

HPLC was used to show rate of chlorothalonil biotransformation over time by the overexpression strains (Fig. 4). The peak for chlorothalonil was identified using a signal at 254nm, and the retention time was 7.5 minutes (Fig. 5).

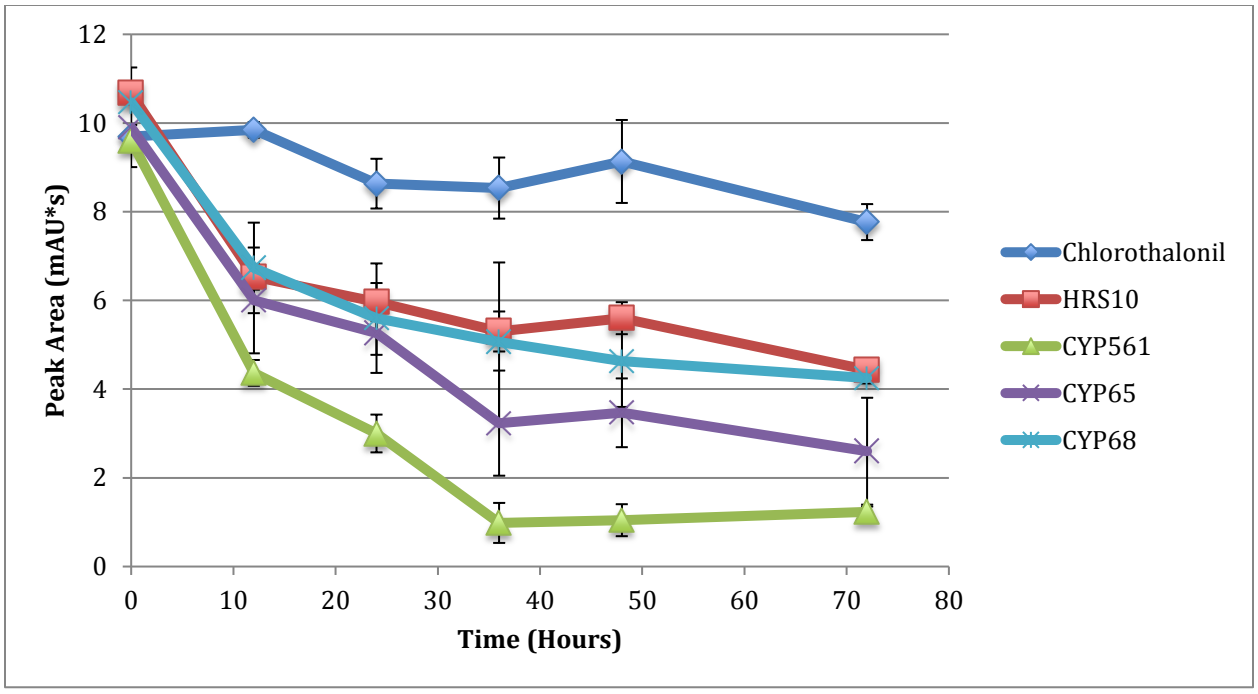


Figure 4. Biotransformation of chlorothalonil over time by CYP450 overexpression strains and DMI sensitive isolate HRS10 by measurement of peak area. Error bars represent standard error.

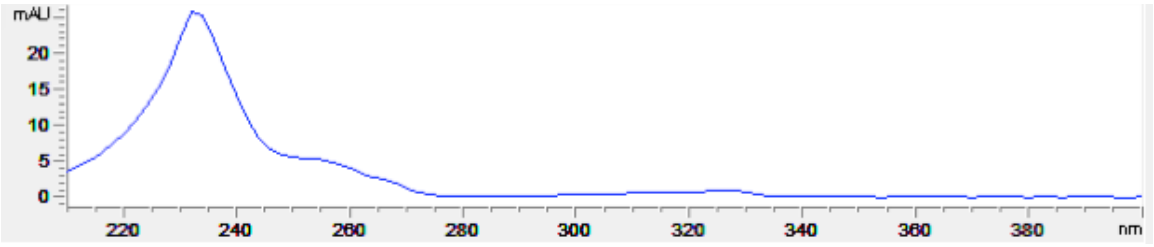


Figure 5. UV spectrum of chlorothalonil.

ShCYP68 did not appear to modify chlorothalonil differently than the wild type, however, ShCYP561 modified chlorothalonil at a significantly faster rate than the wild-type over 72 hours. ShCYP65 was shown to have a slightly faster rate of biotransformation, although not nearly as efficient as ShCYP561.

Identification of the transformation product by GC-MS

The chromatograms for the 4-hydroxy-2,5,6-trichloroisophthalonitrile reference standard and the analytes extracted from HRS10 supplemented with chlorothalonil are shown in Figure 6. The retention times of the main peaks did slightly differ; 11.5 minutes for the reference standard and 10.5 minutes from the extracted sample. This is possibly due to residual media present in the extracted sample.

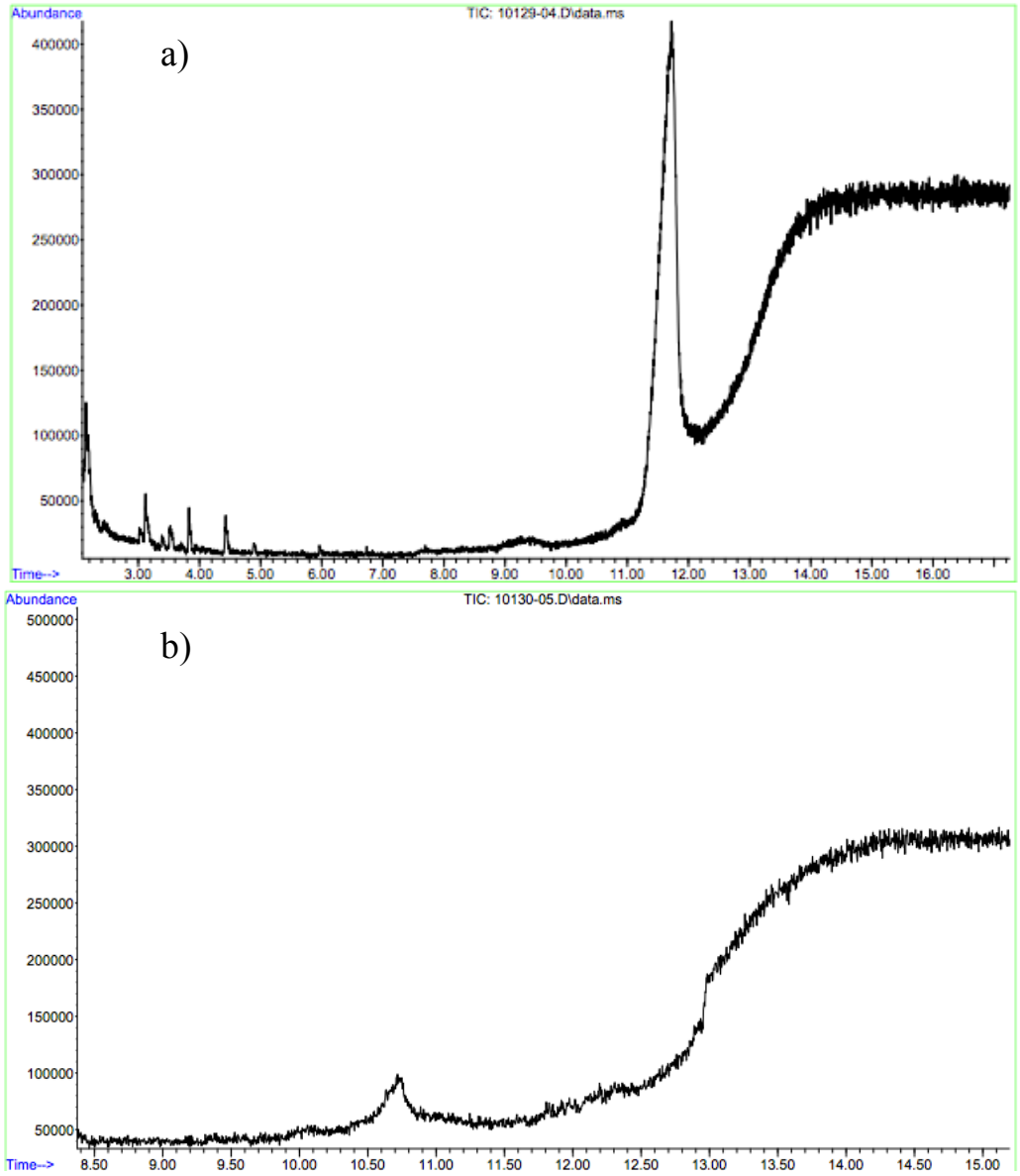


Figure 6. Chromatograms of 4-hydroxy-2,5,6-trichloroisophthalonitrile (a) at ~50 µg ml^{-1}, and the extracted sample from HRS10 (b).

The mass ion for the silylated derivative of 4-hydroxy-2,5,6-trichloroisophthalonitrile (320 MW) was not found (Fig. 7). However, the molecular ion without a methyl group $(M - 15)^+$ was identified, which corresponds well to the findings of Hladik & Kuivila (2008) in their GC-MS analysis of the metabolite. We concluded that *S. homoeocarpa* utilizes CYP450s, specifically CYP561 and CYP65, to biotransform chlorothalonil into the 4-hydroxy-2,5,6-trichloroisophthalonitrile metabolite.

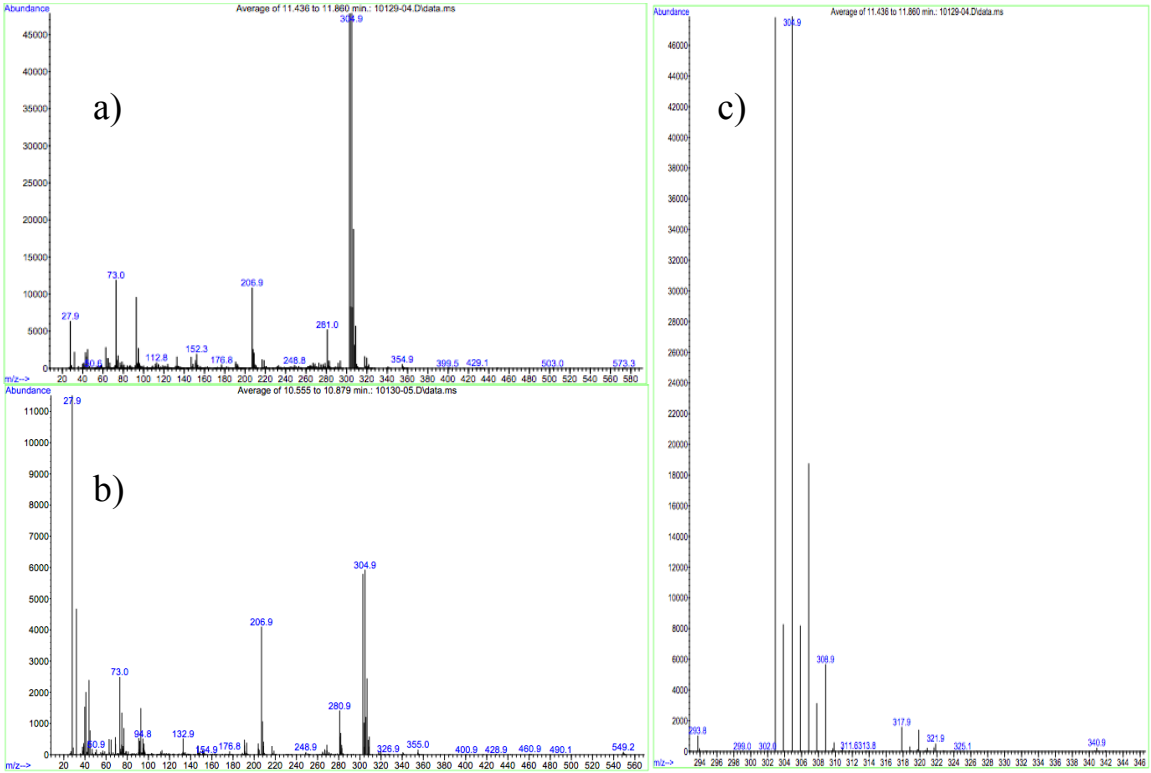


Figure 7. Full mass spectra between 0 and 600 m/z of the 4-hydroxy-2,5,6-trichloroisophthalonitrile reference standard (a), extracted sample from HRS10 (b). A view between 290 and 340 m/z (c) identifies the major m/z fragments of 303, 305, and 307.

Discussion

Xenobiotic detoxification capabilities of ShCYP561, and ShCYP65 and ShCYP68

Many CYP450s play essential roles in primary metabolism, however, it is possible that other CYP450s not directly regulated by ShXDR1 play a role in xenobiotic detoxification, or have multiple functional roles. CYP450s involved in Phase I of xenobiotic detoxification target compounds to increase water solubility, and allow for conjugation reactions to occur. In *S. homoeocarpa,* Sang et al. (unpublished) suggest CYP450s work in tandem with Phase III efflux transporters to gain cross-resistance and multidrug resistance. Here, we show ShCYP561 can oxidize chlorothalonil, although field resistance to chlorothalonil in *S. homoeocarpa* has not been discovered. However, all three CYP450 overexpression strains display a resistance phenotype to propiconazole, with varying in vitro insensitivities to boscalid and iprodione (Sang et al., unpublished). Currently, it is unknown whether or not the CYP450s display substrate specificity to specific fungicide classes. Certain CYP450 families have shown to be highly substrate specific, and other families have shown to be non-specific (Gay et al., 2010; Li et al., 2008). Based on the different chemical structures of the fungicides the CYP450s have been shown to oxidize, ShCYP561, ShCYP65 and ShCYP68 appear to act non-specifically. In addition, our data shows the CYP450s contain much larger cavity sites than their closest homologs, suggesting cavity size might be an important factor for metabolizing larger molecules such as propiconazole. Syed et al. (2013) identified a CYP63A2 enzyme able to oxidize high-molecular-weight polycyclic aromatic hydrocarbons. CYP63A2 has a very large active site cavity, which Syed et al. suggested

plays a role in its non-specificity towards substrates. It is possible that a large active site cavity is a trait of fungal CYP450s involved in xenobiotic detoxification.

At this time, it is unknown whether or not ShCYP561, ShCYP65, or ShCYP68 are involved in other metabolic pathways in *S. homoeocarpa.* Interestingly, only the ShCYP561 overexpression strain had a different growth rate on agar plates. Overexpression of ShCYP561 led to a significantly slower growth rate than the wild-type on minimal media, indicating CYP561 also plays a role in other biosynthetic pathways. In addition, CYP65 and CYP68 displayed the strongest insensitivity to Daconil, however were not shown to biotransform chlorothalonil. This implies CYP65 and CYP68 also play a role in other metabolic pathways, possibly accelerating mycelial growth under stress-conditions.

A novel chlorothalonil biotransformation pathway

Chlorothalonil is a common fungicide primarily applied on peanuts, tomatoes, and turfgrasses (US EPA, 1999). The predominant metabolite found in chlorothalonil treated soils is 4-hydroxy-2,5,6-trichloroisophthalonitrile. The 4-hydroxy metabolite is more water soluble, persistent, and toxic than the parent compound (Cox, 1997). Due to the metabolites' toxic effect and ability to pervade into water systems, more attention has been drawn to the environmental fate of chlorothalonil (Van Scoy & Tjeerderma, 2014). Identification of the 4-hydroxy metabolite was successful by GCMS analysis when HRS10 was cultured in media supplemented with chlorothalonil. Other microbial degradation pathways for chlorothalonil have been identified in bacteria, but to the best of our knowledge, a fungus biotransforming chlorothalonil has not been reported. In

addition, this is the first report of a CYP450 oxidizing chlorothalonil. Since the 4-hydroxy metabolite is the predominant metabolite found in soils, it is possible ascomycete and other fungal CYP450s are important to microbial degradation of chlorothalonil. Whether or not CYP561 and CYP65 are unique in their ability to oxidize chlorothalonil when compared to other fungal ascomycetes is a major question. Searching for a closer homolog to CYP561 or CYP65 in ascomycete species other than the plant pathogenic *S. sclerotiorum* or *B. cinerea* may be revealing.

We hypothesize the reaction mechanism of chlorothalonil biotransformation into the 4-hydroxy metabolite occurs via epoxidation on the 4-position chlorine, and is subsequently reduced to the hydroxyl group by a non-catalyzed reducing agent, such as glutathione. Currently, it is unknown whether *S. homoeocarpa* also initiates a conjugation reaction of the 4-hydroxy metabolite. Conjugation reactions covalently attach smaller polar groups to metabolized xenobiotics after CYP450 action, which produce easily excretable or inactive compounds (Jancova et al., 2010). One ongoing study is characterizing a glutathione S-transferase (GST), which is induced by the DMI fungicide propiconazole based on RNA-seq (unpublished data, Sang et al.). It is also unknown whether or not Phase II enzymes play a significant role in the detoxification system used by *S. homoeocarpa*.

Our investigation of the CYP450s revealed a novel mechanism of chlorothalonil biotransformation. This discovery may be useful in identifying the holistic picture of chlorothalonil's environmental fate. In addition, resistance to chlorothalonil has not been reported in *S. homoeocarpa*, but reduced chlorothalonil field efficacy has been anecdotally reported by golf course superintendents (personal communication, Jung).

Reduced chlorothalonil field efficacy could be due possibly biotransformation of chlorothalonil, or to a multitude of environmental conditions that vary from site to site. In other plant pathogenic fungi, multidrug and chlorothalonil resistant strains of *B. cinerea* have been reported in the field (Barak & Edgington, 2009). It is possible unrelated CYP450s in *B. cinerea* are responsible for the MDR phenotype. In addition, preliminary biotoxicity assays have identified multiple plant extracts with increased antifungal efficacy after inoculation with the CYP450 overexpression strains. Biotransformed intermediates with increased toxicity are a common concern in drug design (Snawder & Lipscomb, 2000), however biotoxification of antifungals by CYP450s has not been studied in ascomycete fungi. It is possible CYP450s are increasing the hydrophilicity of antifungals contained in oils, resulting in increased water solubility and greater bioavailability to the target.

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