ABSTRACT

Title of Thesis:	IDENTIFYING HIGHLY CONSERVED PATHOGENICITY GENES IN CHESTNUT BLIGHT AND POWDERY MILDEW FUNGI AS TARGETS FOR NOVEL FORMS OF HOST RESISTANCE
	Bruce J. Levine, Master of Science, 2019
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A bioinformatic search of the genomes of chestnut blight fungus, *Cryphonectria parasitica* (*Cp*), and the Arabidopsis powdery mildew fungus, *Golovinomyces cichoracearum* (*Gc)*, yielded six suspected pathogenicity genes with homologues in both species. Deletion of these genes by homologous gene replacement was attempted in *Cp*, with one success, *TG4*. The *TG4*-knockout strain showed changes in phenotype and reduced fungal virulence against chestnut. *TG4* appears to be a promising target for host-induced gene silencing (HIGS) in transgenic American chestnut. The use of homologues from genetically tractable species like *Cp* can help overcome the obstacles to performing reverse genetics on intractable, biotrophic fungi such as *Gc*. Experiments underway involving the silencing and ectopic overexpression of the *Gc* homologues of the target genes provide a rapid method to study *Cp* genes, including to screen additional candidate genes as future targets for HIGS.

IDENTIFYING HIGHLY CONSERVED PATHOGENICITY GENES IN CHESTNUT BLIGHT AND POWDERY MILDEW FUNGI AS TARGETS FOR NOVEL FORMS OF HOST RESISTANCE

by

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Dedication

To my wife, Laura Goertzel, who, when I said “I think I want to quit my job at age, 52, even though our children are still young, and go get a degree in a subject in which I have no formal background, “ replied, “I think you should.” Thanks for your support, then, now and forever.

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This thesis, and the path of discovery that it describes would not have been possible without the help of many others.

Dr. Shunyuan Xiao took me on and accommodated my very particular interest in chestnut blight. He helped me craft and carry out a research plan that would potentially contribute to the restoration of the American chestnut, while also building on the Xiao lab's work mapping and characterizing the molecular interactions between plants and their fungal pathogens. I am very grateful to my fellow lab members, Qiong Zhang, Harley King, Xianfeng Ma, Yuheng Yang, Lili Wang, Xiaoting Chen, and Frank Coker, and especially to Ying Wu for her work on selecting the target genes, and other bioinformatics aspects of the project.

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List of Abbreviations

APHIS	Animal and Plant Health Inspection Service
CBA	Chestnut bark agar
CHV	Chestnut hypovirus
CIM	Chestnut induction medium
Cp	*Cryphonectria parasitica*
DLP	Dicer-like protein
dsRNA	Double-stranded RNA
EHM	Extra-haustorial membrane
EPC	Endothia parasitica complete
ER	Endoplasmic reticulum
ET	Ethylene
ETI	Effector-triggered immunity
Gc	*Golovinomyces cichoracearum*
GFP	Green fluorescent protein
HIGS	Host-induced gene silencing
hph	Hygromycin phosphotransferase
HR	Hypersensitive response
IBBR	Institute for Bioscience and Biotechnology Research
JA	Jasmonic acid
mRNA	Messenger RNA
NCL	Normalized canker length
PDA	Potato dextrose agar

PM	Powdery mildew
PTI	PAMP-triggered immunity
RNAi	RNA interference
SA	Salicylic acid
siRNA	Small interfering RNA
RISC	RNA-induced silencing complex
WA	Water-agar

Chapter 1: Research Narrative

Introduction

The long-term goal of this thesis research project is to expand our understanding of the molecular interactions between plants and plant-pathogenic fungi for developing host resistance. We sought to take advantage of two distinct pathosystems to identify key fungal genes, characterize their potential roles in pathogenesis, and explore host-induced gene silencing (HIGS) to develop antifungal resistance in plants. The first pathosystem is the interaction between American chestnut (*Castanea dentata*) and a necrotrphic pathogen *Cryphonectria parasitica* (*Cp*) which results in the devastating chestnut blight disease. The second pathosystem is the interaction between *Arabidopsis thaliana* and a biotrophic pathogen, *Golovinomyces cichoracearum* (*Gc*), one species which leads to a common disease called powdery mildew. Specifically, homologous recombination-based gene replacement will be used to delete conserved fungal genes in *Cp* to investigate their potential role in fungal survival and pathogenesis. Time permitting, homologous *Gc* genes encoding candidate secreted effector proteins (CSEPs) may be expressed in Arabidopsis to test if they can suppress host immunity. Candidate fungal genes identified will be targeted in *Gc* via HIGS using Arabidopsis. Finally, HIGS would be deployed to target essential *Cp* genes identified to engineer *Cp* resistance in American chestnut

Background and Literature Review

Interactions between plants and pathogenic fungi

The study of plant disease is as old as agriculture itself. For thousands of years, humanity has tried to prevent or mitigate the effects of disease on crops through cultural practices and the artificial selection of resistant cultivars. Only recently however, have we begun to understand the biological, biomolecular and genetic basis of plant disease resistance and susceptibility. Key discoveries since the 1950s have made it possible to discover the details about how plants defend themselves from pathogens, and how pathogens can overcome plant defenses in order to parasitize their hosts (Bent 2018). More recent advances in genetics and molecular biology have given us new tools to explore the nature of plant disease more deeply, and continually help us refine our understanding of plant disease.

A certain degree of basal resistance to fungi, bacteria, oomycetes, nematodes, (as well as to herbivores and abiotic stress) is found in all plants. In nature, most plants are resistant to most potential microbial pathogens thanks to the existence of robust preformed and inducible defense barriers (Bent 2018). Preformed (or constitutive) defense barriers of plants include the cellulosic plant cell wall, surface waxes, and other protective compounds and structures that make living plants an inhospitable environment for all but the most adapted microbes (Bigeard 2014). “Plant pathogens” is a term that refers to the subset of microbes that have evolved to produce enzymes, toxins and/or physical structures that enable them to overcome these constitutive defenses, penetrate host plant tissues and derive nutrients from them.

The induced defenses that plants use against broad categories of microbes, serve as a second tier of defense against the specialized, plant-adapted pathogens that overcome plants' constitutive defenses. Pattern recognition receptors (PRRs), proteins embedded in plant cell membranes, can detect compounds which are commonly associated with broad categories of potential pathogens (Boutrot and Zipfel 2017). These pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) include signature compounds of fungi and bacteria, such as chitin and flagellin. Compounds released by damage to the plant cell wall (damage associated molecular patterns, or DAMPS) can also trigger PRRs. When microbes can get past a plant's constitutive defenses and establish themselves on, or damage the plant surface, they trigger a defensive response, generally known as PAMP-triggered immunity (PTI) or MAMP-triggered immunity. In PTI, microbes or damage trigger the PRRs, which in turn initiate chemical signaling cascades between the plant cell plasma membrane and the nucleus (Bigeard 2014). This results in the mobilization of various energy-intensive but highly effective defense responses on the part of the plant, including the production or increased production of anti-microbial compounds, and secretion of enzymes involved in reinforcement and repair of the plant cell wall (Bent 2018, Boutrot and Zipfel 2017).

Plant pathogens have also evolved ways to overcome PTI. They secrete proteins and other compounds, collectively known as effectors, which can disrupt the PTI response. Effectors that pathogens use to disrupt PTI also vary widely in architecture

and function, and tend to be host-specific, allowing a given effector to overcome the defenses of just one or several closely-related plant host species (Kim *et al*, 2018, Jones and Dangl 2016, Lo Presti 2015).

Through the process of attack and counter-attack, plants have evolved a third tier of defense known as effector triggered immunity (ETI). In ETI, plant immune receptors (often called resistance or R proteins) can recognize the presence or virulence activity of certain specific effectors and subsequently trigger even more robust defensive responses in plants (Jones and Dangl 2016). Unlike in PTI, plant immune receptors that detect pathogen effectors are generally intracellular and belong to a nucleotide-binding and leucine-rich-repeat (NB-LRR) superfamily highly conserved across all plant species. During ETI, an R protein gets activated upon recognition of a specific pathogen effector, which leads to elevated biosynthesis of salicylic acid (SA) and production of anti-microbial compounds. ETI is often, although not always, associated with the hypersensitive response (HR). HR is a form of programmed cell death at the site of infection, which can stop some pathogens, especially biotrophs such as powdery mildew, by depriving them of the resources they need to survive (Bent 2018, Jones and Dangl 2006).

In addition to the tight regulation of PTI and ETI by various molecular components downstream of the respective immune receptors, plant defenses are also modulated by phytohormones, such as SA, jasmonic acid (JA) and ethylene (ET). These hormones are also involved in systemic acquired resistance (SAR) and induced systemic

resistance (ISR). SAR and ISR are broad spectrum forms of resistance induced by local infection by microbes, including avirulent fungal endophytes and beneficial root-colonizing bacteria. Kuhn *et al* reported that basal defenses against fungal pathogens, at least in Arabidopsis, rely primarily on PRR signaling to block penetration, but that phytohormone-mediated signaling comes into play once the pathogen has penetrated the plant surface (Kuhn *et al* 2016).

Every pathogen-host relationship (pathosystem) is unique and involves multiple, sometimes hundreds, of molecular interactions at the cellular level, governed by genes accumulated over millions of years of co-evolution (Kim *et al* 2018). The co-evolution can be compared to an arms race that endows both sides with arsenals of molecular weapons and defenses, developed in response to each other over time, through a series of incremental genetic changes. This gradual change allows successful pathogens and hosts to remain in ecological equilibrium with each other. Our knowledge of the nature of these molecular interactions remains incomplete, however, even for highly-conserved interactions common to all plants. A better understanding of these molecular interactions will enable us to understand the disequilibrium that leads to specific diseases and develop strategies to control them.

The demise of American chestnut as a case study in host – pathogen disequilibrium

Background:

The near complete eradication of the once-prevalent American chestnut from the eastern forests of North America, which began in the early 1900s, is a perfect case

study in host-pathogen disequilibrium. The causal agent of chestnut blight, the ascomycete fungus *Cp*, originated in Asia (Rigling and Prospero 2018, Gruenwald 2012), and researchers have described its accidental introduction to North America as a classic example of disasters that can arise when pathogens are introduced to new environments or host species (Anagnostakis 1987). The arsenal of offensive genes *Cp* had developed through eons of co-evolution with Asian chestnut species allowed it to decimate the nearly defenseless American chestnut population, that had never been exposed to it, within a few decades of introduction. *Cp* has been endemic in the American chestnut's entire natural range since the mid-1900s (Rigling and Prospero 2018, Steiner 2017).

Asian *Castanea* species, including Japanese chestnut (*C. crenata*), Chinese chestnut (*C. mollissima*), and two other Chinese species, *C. henryi*, and *C. seguinii,* show variable but generally high levels of resistance to *Cp* (Steiner 2017, Zhang 1998). *Cp* is a minor, superficial disease in Asian chestnut forests, and only causes significant damage when trees are stressed by anthropogenic or environmental factors (Zhang 1998). These Asian chestnut species can also survive in good health in North America due to their blight resistance, but have not replaced American chestnut in the wild during the century since blight arrived, perhaps because they are not ideally adapted to American forests. Asian chestnut species are generally smaller than, and lack the timber form of American chestnut (Schlarbaum *et al* 1992). American and European chestnut species, including *C. dentata*, Alleghany chinkapin (*C. pumilla*), Ozark chinkapin (*C. ozarkensis*) and European chestnut (*C. sativa*), none of which

co-evolved with *Cp*, show variable but low levels of resistance to the fungus (Prospero and Rigling 2017).

The molecular basis for *Cp* virulence and host resistance is poorly understood.

Despite over 100 years of research, the genes that are responsible for resistance in Asian chestnut species have not yet been identified (Steiner 2017). Efforts under way to restore American chestnut to North American forests take various approaches that do not require specific knowledge of naturally-occurring, resistance genes. These include:

- selective intercrossing between the most resistant surviving American chestnuts (Griffin 2006),
- the use of the chestnut hypovirus (CHV), a mycovirus, as a biocontrol against *Cp* (Milgroom and Cortesi 2004),
- backcross breeding of Chinese or Japanese chestnut to the American chestnut background (Steiner 2017, Hebard 2005 and 2014),
- and the use of biotechnology to introduce novel forms of resistance into the American chestnut background (Newhouse *et al* 2014).

Except for the biotechnology approach, in which an oxalate oxidase gene from wheat was inserted in the chestnut genome (Newhouse *et al* 2014), these approaches rely on the introgression of naturally occurring resistance genes from Asian populations into a blight-susceptible American population. Though the loci, sequences, and functions of these naturally occurring resistance genes remain elusive (Steiner 2017),

researchers have uncovered some clues about the mechanisms behind the resistance of Asian chestnut species. Studies of the inheritance pattern (Hebard 2005 and 2014) of blight resistance in Chinese-American hybrid trees, for example, support the hypothesis that Chinese chestnut's resistance to *Cp* involves multiple genes which each contribute partially to resistance.

Several researchers have also isolated compounds or chemical fractions present in Chinese chestnut bark that inhibit fungal growth (Gao and Shain 1995, McCarrol and Thor 1979, Samman *et al* 1979), suggesting a difference in pre-formed or constitutive defenses between Chinese and American chestnut. Studies on the histology of Chestnut blight infections in susceptible and resistant trees, however, also point to induced responses that differ in amplitude between resistant and susceptible trees. Chinese chestnut and resistant hybrids show an ability to contain the spread of *Cp* infection through the rapid lignification of wound periderm around the infection site, while more susceptible trees are slower to lignify, allowing the mycelial fan of the fungus to grow through the defensive wound periderm (Hebard *et al* 1994).

Studies of gene expression in *Cp*-infected and non-infected American and Chinese chestnut tissues (Barakat *et al* 2009 and 2012) are consistent with the findings of Hebard *et al* (1994). Upon infection with *Cp*, Chinese chestnut shows high levels of expression of defense-related genes, followed later by a much smaller spike in metabolic genes associated with tissue repair. In American chestnut, on the contrary, the amplitude of increased expression of defense-related genes is relatively low, but

the subsequent spike in metabolic/repair-associated genes is much higher, likely because the damage done by the pathogen is greater. This suggests that even though Chinese and American chestnut can detect fungal infection, but that the PTI response of Chinese chestnut is faster or more effective, and/or that ETI plays a stronger role in the defenses of Asian chestnut species than it does in *C. dentata*.

Cp can grow as a saprophyte on numerous woody plant species, and it can be a significant pathogen on certain oak species which, like chestnut, are members of the *Fagaceae* family (Roane *et al* 1986). However, the fungus is only severely pathogenic on chestnut species (the *Castanea* genus). This suggests that the highly conserved, broad-spectrum, constitutive and PTI defenses found in non-host plants are sufficient to protect them from *Cp*, and that the pathogen has adapted to produce effectors that can disable or bypass the particular forms of these defenses that are specific to the *Castanea* genus and its closest relatives. The fact that *Castanea* species that co-evolved with *Cp* are resistant, but isolated populations such as *C. dentata* are not, also suggests that ETI developed in Asian chestnut species under the selective pressure of *Cp*.

Most of our current knowledge about the molecular interactions between *Cp* and chestnut comes from investigation of the naturally-occurring mycovirus CHV, which has effectively prevented the eradication of the *C. sativa* in Europe (Rigling and Prospero 2018). CHV infects *Cp* and can significantly reduce its virulence. The capsidless virus is widespread in Europe and Asia, and is transmitted horizontally by

anastomosis between mycelia, and vertically through asexual spores (but not sexual spores). Research into viral hypovirulence has revealed several *Cp* genes suppressed by the virus which are essential for virulence, some of which appear to have regulatory functions and others of which (summarized in table 1 below) appear to encode proteins directly involved in pathogenesis. These genes are involved in the biosynthesis of secreted enzymes or phytotoxins, and many are co-regulated through *Cp*'s G-protein signaling pathway (Dawe *et al* 2004).

Table 1. *Cp* genes identified through research into CHV as being involved in fungal pathogenicity or virulence in chestnut.

Gene	**Putative function**	**Reference**
CHB1	Cellobiohydrolase involved in breakdown of cellulose	Wang and Nuss 1995
CRP	Cryparin, an abundant *Cp* hydrophobin associated with fruiting body eruption	Zhang *et al* 1994
KEX2	Protease necessary for virulence but not vegetative growth	Jacob-Wilk *et al* 2009
LAC3	Extracellular laccase (phenoloxidase) necessary for virulence	Chung *et al* 2008 Kim *et al* 1995
PRB1	Subtilisin-like protease involved in both vegetative growth and virulence	Shi 2014
OAH	Oxaloacetylhydrolase enzyme necessary for the production of the phytotoxin oxalic acid	Havir and Anagnostakis 1985

While virulence factors revealed by CHV could serve as targets for engineered forms of resistance to *Cp*, they do not appear to be effector proteins, and are not necessarily responsible for the *Cp*'s unique pathogenicity on Chestnut. They appear to support fungal processes which are necessary, but not sufficient by themselves, for *Cp* to colonize and severely parasitize live chestnut tissue. For example, the *Cp OAH* gene

is essential for production of the secreted phytotoxin oxalic acid, and the suppression of this gene renders *Cp* avirulent against chestnut (Havir and Anagnostakis 1985). There are, however, numerous fungi that produce oxalic acid (Dutton 1996), among which only *Cp* is a significant chestnut pathogen. Most likely, *Cp*'s ability to invade chestnut tissue and kill cells with oxalic acid is only possible because the fungus also produces as-yet-unidentified effectors that overcome chestnut's constitutive and PTI defenses.

Improved sequencing technology, and the recent completion of an American chestnut genome (Schmutz *et al*, not yet published) will help researchers identify and characterize some of the natural resistance genes in highly-resistant Chinese-American hybrid chestnuts (Steiner 2017, Westbrook 2018). This information will greatly improve the efficiency of programs to breed for resistance. However, even if such programs are successful in capturing and fixing major Chinese chestnut resistance genes in a mostly American hybrid population, there is reason to believe that this may not bring about equilibrium between host and pathogen. Zhang *et al* (1998), in their analysis of the dynamics between *Cp* and the blight-resistant *C. molissima* in Chinese chestnut forests, describe the relationship as a "hybrid system" in which the disease is kept under control by both host resistance and CHV infection of the fungus. Though CHV has been released, and also found to occur naturally in North America, it has not become a widespread or durable biocontrol as it has naturally in Europe or Asia. The reasons for this remain poorly understood (Milgroom and Cortesi 2004). It is therefore likely that novel forms of resistance

will be a necessary to supplement the introgression of naturally occurring Asian resistance genes in the overall effort to restore the American chestnut.

Rationale and significance of this research

The growing importance of molecular genetics for crop protection and environmental integrity

The population of the world is projected to reach 9.7 billion in 2050 (United Nations 2017). This will put great pressure on a global agricultural system that has limited new land to put under cultivation, and which already relies heavily on non-renewable fossil fuels and diminishing fresh water sources to meet current demand. To meet the challenge of increasing agricultural yields, an obvious priority is to reduce the amount of food lost to plant disease. Currently, an estimated 30% of crops planted are lost to pre- or post-harvest disease, with plant-pathogenic fungi accounting for most of this loss (Bent 2018). Climate change and human behavior are exacerbating this problem by bringing crops and wild plants into contact with pathogens which have not previously threatened them (Cline 2007).

Despite advances in biotechnology and genetic engineering, most improvements in resistance to pathogens of economically important plants still rely on traditional breeding to capture naturally occurring genetic resistance (Chrispeels 2018). There is no guarantee that this approach will remain sufficient to stay ahead of accelerating

changes in plant-pathogen dynamics, a problem which has implications for food security and for environmental integrity. New developments in biotechnology, however, offer the possibility of developing novel forms of disease resistance for both crops and endangered wild plants, and to protect and improve agricultural productivity and ecosystem integrity. Realizing the potential of biotechnology to combat fungal disease threats will require a better understanding of the molecular interactions between pathogenic fungi and their plant hosts.

The Arabidopsis-powdery mildew model pathosystem

Many important discoveries about plant defenses against fungal and other microbial pathogens have been achieved through studies of model species, such as Arabidopsis, the first plant to have its genome fully sequenced. In addition to the availability of a reference genome, Arabidopsis also offers the advantages of a short life cycle (as little as 6 weeks from seed to seed), being easy to self- or cross-pollinate, and amenability to genetic modification. Arabidopsis is also susceptible to certain species of powdery mildew (PM) fungi, and there are mutant Arabidopsis lines that show either resistance to PM species that are well-adapted to Arabidopsis, or susceptibility to PM species that are poorly adapted to Arabidopsis wild-type plants. Forward genetic studies of Arabidopsis have thus enabled researchers to identify Arabidopsis genes associated with resistance or susceptibility to PM. This has revealed important details about the molecular interactions that either allow or prevent PM infection in Arabidopsis, and homologues of such host components are found in other plant species.

Two important examples of discoveries that have emerged from examination of the PM-Arabidopsis pathosystem are *RPW8*-mediated broad-spectrum resistance, and loss-of-*MLO (mlo)*-mediated complete resistance against PM fungi. *RPW8.2*, a member of a small family of broad-spectrum resistance genes identified and characterized by Xiao *et al* (1997 and 2001), encodes a resistance protein that is targeted to, and functions at the extrahaustorial membrane (EHM), a host-derived membrane that surrounds the PM feeding structure known as the haustorium. The EHM is the principal interface between host and pathogen, and the primary place at which effector proteins enter host cell and nutrients are taken up by the fungus. RPW8-mediated resistance is SA-dependent, involves the accumulation of hydrogen peroxide, and can lead to HR response in the infected cell (Xiao *et al* 2001). *mlo*-mediated broad-spectrum and durable resistance to PM fungi in barley has been employed in agriculture for close to a century (Buschges *et al* 1997, Piffanelli *et al* 2004). The Arabidopsis *MLO2* gene, along with its close homologs *MLO6* and *MLO12* plays a similar role in PM penetration of host cells (Consonni *et al* 2006). Mutant plants with non-functional *MLO2* or multiple non-functional *MLO* family genes show strong resistance to penetration by PM fungi (Kuhn *et al* 2016). However, the molecular basis of both *RPW8*- and *mlo*-mediated resistance to PM fungi remains to be elucidated (Kuhn *et al* 2016).

One of the main reasons that little is known about *RPW8* and *mlo*-mediated resistance from the pathogen's perspective is because PM fungi are genetically intractable. To

date, no one has perfected any method to make stable, targeted mutations in the genomes of biotrophic fungi such as PM. This means that reverse genetic methods, such as gene deletion and gene over-expression can only be applied to the host in the PM-Arabidopsis pathosystem. Genetic exploration of the pathogen is very limited in the case of Arabidopsis-PM interaction, and the genetic basis of fungal virulence in PM fungi consequently remains poorly understood.

The merits of the chestnut-Cp *model system*

Unlike biotrophic PM fungi, *Cp* as a nectrotrophic pathogen is genetically tractable (Nuss 2011). Reverse genetic studies with *Cp* have allowed researchers to conduct knockout studies for candidate genes and identify essential factors in fungal virulence in chestnut (table 1). There are several advantages of using *Cp* as a model species for studying fungal virulence or pathogenicity. These include: (1) *Cp* can be easily cultured in vitro and stored at -20°C for many years; (2) the virulence of *Cp* fungal strains in chestnut can be quickly assessed through various types of controlled inoculations; (3) vegetative *Cp* spores and mycelia are haploid, resulting in phenotypic expression of relevant mutations; (4) *Cp* quickly produces uni-nucleate, haploid asexual spores in culture, making it possible to isolate monokaryon knockout strain mycelia through a variety of screening methods; and finally (5) the availability of DK80, a mutant strain of *Cp*, which is highly virulent but has an impaired non-homologous end joining capability, makes genetic transformation of the fungus by homologous recombination highly efficient (Nuss 2011). In addition, a well-annotated reference genome is available for the standard virulent *Cp* strain EP-155

(U.S. Department of Energy, Joint Genome Institute, https://genome.jgi.doe.gov/Crypa2). By using this ascomycete fungus as a surrogate for related biotrophic fungi, such as PM species, we can overcome some of the limitations on performing reverse genetic studies with biotrophs, at least for the rapid screening of candidate genes with homologues present in both species.

Defining pathogenicity broadly for target gene selection

This study seeks to identify genes that are highly conserved in ascomycete plant pathogenic fungi and play a role in fungal pathogenesis in plant hosts. Our focus goes beyond fungal gene products that act on the plant host, such as digestive enzymes, toxins and effector proteins, and covers a broader concept of pathogenicity that includes internally-acting fungal genes that enable fungi to survive in the well-defended, hostile and often nutrient-poor environment of a live host. We also emphasize candidate genes that are highly conserved across species, and we do so for two reasons: (1) that we will be able to use reverse genetic methods (particularly gene knockout) to explore the function of genes that exist in a genetically tractable fungus (*Cp*) to identify important virulence factors for further study in intractable biotrophic fungus (*Gc*); and (2) that it may lead to the development of novel forms of resistance that may be applicable to multiple plant-pathogenic fungi. More immediately, the information we obtain from the *Cp* – chestnut pathosystem through genetic modification of the *Cp* fungus may be useful for us to functionally characterize candidate homologous PM genes through host-induced gene silencing (HIGS).

HIGS is a relatively recently developed method for engineering plants with resistance to specific pathogens. HIGS relies on RNA interference (RNAi), a process that occurs in eukaryotic cells (Weiberg *et al* 2014.) In RNAi, a host cell produces enzymes known as dicer-like proteins (DLPs), which cleave double stranded RNA (dsRNA) molecules into 21-22 base pair short fragments, known as small interfering RNAs (siRNAs). These siRNA fragments are then incorporated into RNA-induced silencing complexes (RISCs), which are also produced by the host cell. The siRNA fragments in the RISCs serve as template to bind complementary messenger RNA (mRNA) molecules and guide the RISCs to cleave the target mRNA and /or disrupt its translation, thus silencing expression of the gene from which the mRNA was transcribed (Weiberg *et al* 2014).

Though RNAi was originally understood as a defense against dsRNA viruses and/or a process for regulating host gene expression, it is now known that there is also two-way trafficking of dsRNA between pathogens and plants (Weiberg *et al* 2014, Baulcombe 2015, Han and Luan 2015). There are fungal pathogens that export dsRNA to plants where they silence host genes, and plants that export dsRNA to silence pathogen genes (Cai *et al* 2018). HIGS technology involves the artificial insertion of genes into a host that encode dsRNA matching target genes from a pathogen's genome. These dsRNAs can be trafficked into the pathogen where they help silence the target gene. Though the silencing is not always complete, HIGS can at least down-regulate target genes. It can be used experimentally, as a substitute for gene knockout, or as a novel form of defense in genetically modified plants. HIGS-based defenses have been successfully demonstrated in several plant species,

including in papaya against ring spot virus (Gonsalves 1998), in barley against Fusarium head blight fungus (*Fusarium graminearum*) (Nowara *et al* 2010, Koch *et al* 2013), and in banana against Panama disease (*Fusarium oxysporum f.sp. cubensis*) (Ghag *et al* 2014).

Research objectives

This research examines the function of six highly conserved genes found in plant-pathogenic fungi, focusing on the roles they play, with a view to discovering new targets for fungal gene disruption that could be employed in novel forms of defense. The six genes in this study have homologues in *Cp* and six PM species, including the Arabidopsis pathogen *Gc*, the genomes of which have been sequenced and annotated (Wu *et al* 2018). We screened homologous genes found in *Cp* and *Gc*, emphasizing candidates whose PM homologues are upregulated in the haustorium and likely to be involved in pathogenicity. (Note: gene selection methodology and results are discussed in greater detail in chapter 2). We attempted to delete these genes, by homologous gene replacement in *Cp*, and observe the effect on the fungus *in vitro* and *in planta*. After studying the selected genes in the *Cp*-chestnut pathosystem, we intend to silence their homologues in Arabidopsis by means of HIGS, and compare the effect on fungal virulence to that observed in *Cp*. This research is exploratory in nature and is intended to generate additional testable hypotheses about specific genes or types of genes that may serve as targets for developing novel forms of defense against fungal pathogens in genetically modified plants.

The research presented in this thesis has 4 main objectives:

1. To test the concept of using a genetically tractable necrotrophic pathogen as a surrogate for reverse genetic studies of homologous genes in a genetically intractable, biotrophic pathogen,
2. To functionally characterize previously unstudied fungal genes for their potential roles in pathogenesis,
3. To improve our understanding of molecular interactions between hosts and pathogens in the Arabidopsis-PM pathosystem, and
4. To identify *Cp* genes as targets for HIGS-mediated resistance to *Cp* in American chestnut

Chapter 2: Genetic Study of six Highly-Conserved Genes in *Cp*

Introduction

The Chestnut-*Cp* interaction and Arabidopsis-*Gc* interaction, each have distinct features. While *Cp* is amenable to genetic manipulation (Nuss 2011), genetic modification of chestnut trees is difficult and time consuming (Newhouse et al 2014). Conversely, while Arabidopsis is genetically amenable, *Gc* is not. In this work, we sought to use both pathosystems in a complementary. To bypass technical barriers that prevent reverse genetic research on biotrophic fungi such as *Gc*, we targeted homologues of *Gc* genes found in the genetically transformable necrotrophic plant pathogen *Cp*. Any of these homologous genes whose deletion in *Cp* results in reduced virulence in its host (chestnut) would not only be promising targets for novel forms of resistance to chestnut blight, but also candidates as possible pathogenicity genes in *Gc*. While targeted mutagenesis/gene knockout is not possible in *Gc*, HIGS could be used to suppress gene expression. The first step was to identify promising target genes.

Materials and methods

Identification of target genes

Our criteria for selecting homologous gene pairs across the two ascomycete fungal species (*Cp* and *Gc*) included high-level protein sequence homology ($E<10^{-6}$), the presence of a predicted N-terminal signal peptide, the absence of predicted transmembrane domains, and increased expression of the *Gc* homologues in haustoria

(Wu *et al* 2018). We rejected genes whose homologues had been shown to be essential for survival (i.e. lethal when deleted) in the model ascomycete yeast *Saccharomyces cerevisiae (Sc)*. These criteria were designed to capture a broad range of secreted proteins involved in colonization of, and adaptation to, the host, rather than just those that resemble proteins involved in known pathogenesis pathways. We did not include in our selection criteria information about putative gene function, or other characteristics generally considered typical of effector proteins or pathogenesis-related genes, such as short protein sequence, cysteine richness or lack of homologues outside of *Gc* and *Cp* (Kim *et al* 2016).

Target gene selection began with browsing the genome of *Gc* strain UCSC1 (Genbank accession number MCR00000000.1), using SignalP3.0 (www.cbs.dtu.dk/services/SignalP-3.0/) to predict potential N-terminal secretion signal peptides and TMHMM 2.0 (www.cbs.dtu.dk/services/TMHMM) to predict transmembrane domains in the mature peptides. We then did BlastP searches of the resulting list of *Gc* genes against the genome of *Cp* strain EP155 (U.S. Department of Energy Joint Genome Institute, genome.jgi.doe.gov/Crypa2). The *Cp* EP155 strain is the parent strain of the *Cp* DK80 mutant strain used for transformation in this project. Simultaneously, we also did a BlastP search for homologues of the same genes in the genome of *Sc* strain S288C (GenBank accession number PRJNA128, ncbi.nlm.nih.gov/bioproject/128), to screen out genes found to be essential in this extensively studied genome.

Using the criteria above, we identified six potential pathogenicity genes with homologues in *Cp* and *Gc*. The six genes were designated as Target Gene one through six (hereafter *TG1-TG6*), in priority order based on the degree to which homologues have been reported in plant-pathogenic fungi, and the levels of amino acid sequence identity between the *Cp* and *Gc* homologues. None of these six genes appear to have been previously studied or characterized in either species. However, BlastP searches of the predicted proteins in *Cp* produced numerous homologues for each, and several of the genes have been characterized or studied in *Sc* or other species of fungi.

Creation of gene disruption constructs

We attempted to develop knockout strains of *Cp* for each target gene using homologous gene replacement techniques described by Churchill *et al* (1990). The *Cp* strain DK80, obtained from Dr. Dongxiu Zhang at the U.S. Department of Agriculture's Agricultural Research Service in Beltsville, MD, is a mutant strain derived from the standard virulent research isolate of *Cp*, EP-155, with a gene essential for non-homologous end joining deleted (Lan *et al* 2008). DK80 can be transformed by homologous recombination with up to 85 percent efficiency (Nuss 2011) by incubating DK80 spheroplasts in an osmotic solution at room temperature with chimeric fragments of DNA whose flanking sequences match targeted portions of the DK80 genome (Churchill *et al* 1990).

Chimeric DNA fragments, to be used as gene disruption/knockout constructs, were designed using Benchling molecular cloning web tools (Benchling.com). One type of

chimeric fragment was developed for each target gene. Each included a common marker cassette from the pKAES173 plasmid that contains a hygromycin resistance enzyme (hygromycin phosphotransferase – hph) controlled by a constitutive fungal promoter from *Aspergillus nidulans*. We obtained the pKAES173 plasmid from the Nuss lab at the University of Maryland's Institute for Bioscience and Biotechnology Research (IBBR). Flanking sequences matching those of the relevant target gene, were fused to the 5' and 3' ends of the marker cassette using overlapping PCR.

Flanking sequences were cut from DK80 genomic DNA using outer forward (FSFPs) and reverse (FSRPs) primers matching 200-600 bp of the 5' and 3' flanking sequences of the target genes and overlapping primers that included one part matching the other flanking sequences fused to 20-22 bp segments complementing the relevant end of the marker cassette. These PCRs resulted in the amplification of target gene flanking sequences with 20-22 bp overhangs matching the marker cassette. The marker cassette itself was amplified from the pKAES173 plasmid by PCR with simple forward and reverse primers (Marker cassette forward and reverse primers – MCFP and MSRP). All primers were designed with the web-based molecular biology platform Benchling (Benchling.com) and manufactured by Eurofins. All PCR reactions were performed with Takara Extaq high fidelity polymerase and Extaq 10x PCR buffer. Primer sequences and melting temperatures are provided in table 2**.**

Table 2. Primers used in the amplification of chimeric gene disruption constructs for *TG1-TG6,* and their melting points. FSFP and FSRP refer to flanking sequence forward and reverse primers. MCFP and MCRP: marker cassette forward and reverse primers. OFP and ORP: overlapping forward and reverse primers. The common sequences between the OFPs/ORPs and the marker cassette¸ and melting points, are indicated in bold.

Primer	5' to 3' sequence	Tm
MCFP	TGCAGCCCGGGGGATCCATAA	62°C
MCRP	CGACGTTGTAAAACGACGGCCA	60°C
TG1-FSFP	AGACTTGCCATTTCTTCTCTCCT	61°C
TG1-FSRP	GCATATGAGTCTTTGAGCAAAACGA	61°C
TG1-OFP	**TGGCCGTCGTTTTACAACGT**CGGGGTACGGCATCAGCGA	**60°C** /54°C
TG1-ORP	**TTATGGATCCCCCGGGCTGC**AGAGTGATGTCGACGTGAAAAGA	**62°C** /55°C
TG2-FSFP	AAGTCAGAGAAGGGGAAAGTGA	64°C
TG2-FSRP	CGGTGGACTTCGGTTGACT	64°C
TG2-OFP	**TGGCCGTCGTTTTACAACGTC**GCTCAACTTCGTCCCTCCGT	**60°C**/55°C
TG2-ORP	**TTATGGATCCCCCGGGCTGCA**GAGTCGAACGGTGTGTCGT	**62°C**/57°C
TG3-FSFP	CCCTCGGTTGCTCAGTATATCA	65°C
TG3-FSRP	GGAAGAAGAGGTG*GC*GGTA	64°C
TG3-OFP	**TGGCCGTCGTTTTACAACGTC**GTCGCAGATCTCTGATGGTAAGTT	**60°C** /55°C
TG3-ORP	**TTATGGATCCCCCGGGCTGCA**CGTTGTTGTTTTGTGGCGTTTA	**62°C** /55°C
TG4-FSFP	ACAAGATGTCGTGGTATTACTAGGA	62°C
TG4-FSRP	GCTTGGAATTTGGTGGTGGA	65°C
TG4-OFP	**TGGCCGTCGTTTTACAACGTC**GTGAAAGGAAAAAGCCAGGTTGA	**60°C** /55°C
TG4-ORP	**TTATGGATCCCCCGGGCTGCA**GCTCCTCCCAGATTGCAGAT	**62°C** /56°C
TG5-FSFP	GCAGGGTGAACCTGATTTCTCTACCACATCAAAAT	63°C
TG5-FSRP	CAGGCAAACAATGCCTGCCAGCTTAT	62°C
TG5-OFP	**TGGCCGTCGTTTTACAACGTC**GTTGGGGGAGGTGGGATCTCAAGTCA	**60°C** /63°C
TG5-ORP	**TTATGGATCCCCCGGGCTGCA**CACCTGATGTTATACAAGACCAAGTGGTTGTCAA	**62°C** /62°C
TG6-FSFP	TAATGTGAGCAGGAGCATCTTGACGAAGTGTTT	63°C
TG6-FSRP	GTGGTTTTAACACTTTACTAGAGGCGCATATTTACCATCATATATTA	62°C
TG6-OFP	**TGGCCGTCGTTTTACAACGTC**GCTATGACTGACAAGTGGACGCCGCT	**60°C** /63°C
TG6-ORP	**TTATGGATCCCCCGGGCTGCA**TTTCTATTGACTTTGAGCAAGTACTCGTGCA	**62°C** /60°C

For each target gene, full chimeric fragments were generated by overlapping PCR using the forward primer of the 5' flanking sequence as a forward primer and the reverse primer of the 3' flanking sequence as a reverse primer. During the PCR, denatured marker cassette segments annealed to the overlapping tails of the flanking sequences resulting in fusion and amplification of all three fragments. A diagram of the chimeric fragment assembly is provided in figure 1. The resulting PCR products were purified through gel electrophoresis in 1% agarose gel and measured with a standard 1 kb DNA ladder (Fermentas Generuler). The bands corresponding to the

full length of the expected chimeric fragment were cut from the gel and purified using a ThermoScientific GeneJET Gel Extraction Kit.

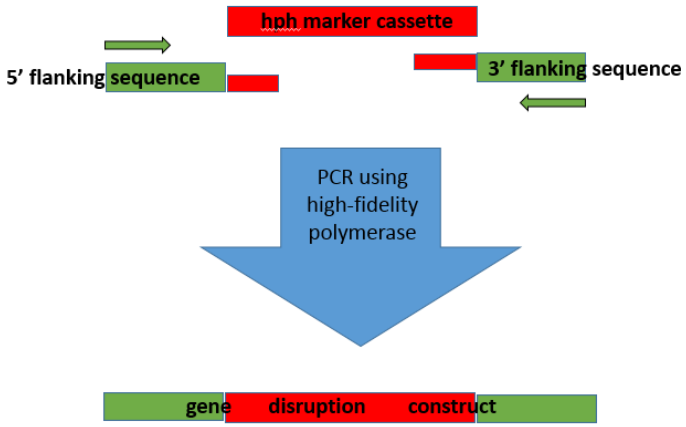


Figure 1. Schematic of the overlapping PCR process to create chimeric gene disruption constructs from separate fragments of DNA.

Concentrations of chimeric gene disruption constructs amplified by overlapping PCR for *TG1-TG4* and *TG6* ranged from 14.6 to 35.2 ng/µl, too low to efficiently produce the 5-10 mg required for fungal spheroplast transformation (Churchill *et al* 1990). To increase concentrations, chimeric fragments for *TG1, TG2, TG3, TG4 and TG6* were inserted into and amplified in *E. coli* bacteria, and plasmid DNA was extracted (ThermoScientific GeneJet miniprep kit). Multiple attempts produced successful results for different target genes using different vectors, bacteria and extraction methods (table 3). Because the DNA concentration of *TG5* overlapping PCR product was relatively high, bacterial amplification was not necessary. *TG5* fragments were produced with overlapping PCR, using 5 cycles without primers to encourage the flanking sequences to anneal to the marker cassette fragments, followed by 30 cycles with the 5' forward and 3' reverse primers added.

Table 3. Methods used for amplification of the *TG1-TG6* gene disruption constructs. The pGEM-T EasyVector and T-4 ligase were purchased from Promega. The pGXT plasmid was obtained from Dr. Guoliang Wang, Ohio State University.

Target Gene	Initial DNA conc. (ng/µl)	Plasmid used for transformation	Bacteria type	Amplification/Extraction method	Final DNA conc.
TG1	18 ng/µl	pGEM-T EasyVector/T4 ligase	Invitrogen OneShot Top10 chemically competent cells	Miniprep	441 ng/µl
TG2	34 ng/µl	pGEM-T EasyVector/T4 ligase	Invitrogen OneShot Top10 chemically competent cells	Miniprep	287 ng/µl
TG3	15 ng/µl	pGEM-T EasyVector/T4 ligase	Invitrogen OneShot Top10 chemically competent cells	Miniprep	278 ng/µl
TG4	18 ng/µl	pGXT plasmid/T4 ligase	Invitrogen OneShot Top10 chemically competent cells	Miniprep, followed by 35 cycles of PCR amplification using ExTaq polymerase	39 ng/µl
TG5	107 ng/µl	n/a	n/a	Overlapping PCR	107 ng/µl
TG6	64 ng/µl	pGXT plasmid/T4 ligase	Invitrogen OnShot Top10 electro-competent cells	Miniprep	98 ng/µl

Spheroplast preparation

Spheroplasts of *Cp* strain DK80 were prepared following the protocol described in Appendix II. DK80 was grown on PDA medium, and aerial hyphae were harvested by swirling through the aerial layer with a pipette tip, accumulating a small sphere (approximately 5 mm in diameter) of hyphal tissue. The sphere was transferred to a 1.5 mLcentrifuge tube, washed with distilled water, and ground with 50 twists of a small plastic micro-pestle. The contents were transferred to 100 mL of potato dextrose broth in a sterile 250 mL Erlenmeyer flask and incubated on a bench top over three days until about half the volume of the medium was occupied by white, cloudlike mycelial masses.

The mycelium was strained and transferred to sterile 50 mL falcon tube, and spun down at 3700 rcf for 5 minutes at room temperature. The mycelial pellet was washed with distilled water, and spun down again at 3700 rcf for 3 minutes, after which the washing step was repeated one more time. The mycelium was removed from the tube and gently blotted on sterile filter paper to remove excess water and then placed and re-suspended in a new sterile 50 mL falcon tube containing 25 mL of digestion buffer, freshly prepared in the same tube. (The protocol for the preparation of digestion buffer, and other buffers and media used in the spheroplast preparation, transformation and regeneration steps is provided in Appendix II).

The mycelium and digestion buffer was incubated horizontally overnight (approximately 16 hours) in a 30°C shaker at 50 rpm, resulting in a homogenous cloudy suspension. After removal from the incubator, cold, sterile trapping buffer was added to overlay the spheroplast suspension until the tube was filled to 50 mL, with care taken not to disrupt the spheroplast layer. The suspension and trapping buffer were spun down at 3,700 x g for 5 minutes at 4°C. Spheroplasts were collected at the interface with a 1,000 µl pipette and transferred to a sterile 50 mL falcon tube, and diluted with two volumes of 1M sorbitol solution, and mixed gently but thoroughly. The suspension was then evenly distributed to 1.5 mL centrifuge tubes, and spun down again at 6,000 rcf at 4°C for 5 minutes. Supernatant was removed by pipette, and the spheroplasts were suspended in a single 200 µl volume of STC, which was transferred from tube to tube to resuspend all of the pellets in all of the tubes in a single volume. The final volume of spheroplasts suspended in STC was

spun down again at 6,000 rcf for 5 minutes. The supernatant was removed and replaced with 1 mL of STC, which formed a slightly cloudy homogeneous solution. Approximately 10 µl of spheroplast suspension was observed on a hemocytometer under a dissecting microscope at 250x magnification. Spheroplasts were counted and concentration was estimated at 2 x 10^6 spheroplasts per mL (figure 2). The solution was then diluted with one part PTC and 0.05 parts DMSO per 4 parts STC, then aliquoted into 1.5 mL tubes and placed in a -80°C freezer for storage in 50 µl units. (Appendix II provides formulations for all buffers related to spheroplast preparation and transformation.)

Figure 2. *Cp* spheroplasts under a hemocytometer.

Spheroplast transformation

Genetic transformation to create *Cp* knockout strains was accomplished by incubation of freshly prepared or thawed spheroplasts in a polyethylene glycol (PEG)-based osmotic buffer containing high concentrations (~10 µg of gene disruption constructs per transformation) at room temperature, using methods described by Churchill *et al* (1990). (The protocol and recipes for all buffers and solutions are provided in Appendix II.)

For each transformation, a DNA solution containing the relevant gene disruption construct was placed in 1.5 mL centrifuge tubes with enough distilled water to reach a volume of 10 µl. Fifty µl of spheroplast suspension was added to each tube, gently mixed and allowed to chill on ice for 30 minutes. Then, 500 µl of PTC was added to each tube, mixed gently and incubated at room temperature for 25 minutes. One mL of STC was then added to each tube to stabilize the osmotic pressure, stop the transformation process, and create a ready-to-use suspension of spheroplasts.

Regeneration of putative transformants

Following the transformation step, the spheroplast suspension was transferred to labelled 85 mm petri dishes in serial dilutions of 2 µl, 20 µl and 200 µl (for first transformation of *TG1-TG4*) or 5 ul, 20 µl and 80 µl (for second and third transformations of *TG1-TG3, TG5-TG6*). One control dish was inoculated with the untransformed DK80 spheroplast suspension. Ten mL of regeneration medium (see Appendix I) was added to each dish and mixed with the spheroplast suspension by

swirling. Dishes were allowed to solidify under the laminar flow hood and covered. After 16 hours of incubation on the benchtop another layer of regeneration medium with hygromycin was added, allowed to solidify and cool, and then covered and moved to incubate on the benchtop. (The hygromyicin concentration in the upper layer of regeneration medium was 50 mg/mL in the first set of transformations of *TG1-TG4*, and then attempted at 30, 40 and 50 mg/mL in subsequent attempts with *TG1-TG3*, *TG5* and *TG6* . The plates were observed for 3-5 days for the emergence of hyphae on the surface of the regeneration medium. Up to twelve small colonies per knockout strain were named with capital letters and then transferred to individual new petri dishes containing potato dextrose agar (Difco Bacto PD broth plus agar - PDA) medium amended with the selective dose of hygromycin (PDA+hyg) and allowed to grow out. The putative transformant mycelia were labelled by knockout strain followed by the letter identifying the specific colony transferred (e.g. TG1A, TG2C).

Plates containing putative transformed mycelia were allowed to grow out on the bench top until the mycelium was large enough to exhibit different morphology the center of the colony than at the growing margin. Two 5 mm punches of each putative transformant and of the untransformed DK80 control were taken from the margin of the mycelium. One of each was transferred to the center of a fresh 60 mm PDA+hyg plate, and another to the center of a 60 mm plate of hygromycin-free PDA. This resulted in two cultures (+hyg and -hyg) of each putative transformant paired with two cultures of the DK80 control (+hyg and -hyg). Each four-plate set was allowed

to grow out on the bench top until the most rapidly growing mycelium of the four approached the edge of the petri dish (generally 3 days). At this point the transformants were photographed and phenotypic observations noted.

Isolation of monokaryon knockout strain mycelia

Tissue from the +hyg culture for each putative transformant and from one DK80 -hyg culture was harvested when aerial hyphae were relatively abundant, and genomic DNA was separately extracted from these samples using a tissue grinder and SDS-based DNA extraction method. Separate PCR analyses were carried out using each sample of genomic DNA, using the forward and reverse primers for the hygromycin resistance marker cassette, and using the flanking sequence primers for each target gene as forward and reverse primers for the whole gene disruption construct. Electrophoresis of the PCR products was carried out in a 1% agarose gel, with a 1 kb ladder (Fermentas GeneRuler) used to gauge DNA fragment size. DK80 samples were used as negative (non-transformed) controls. The presence of a band for PCR reactions using marker cassette primers identified putative transformants. Differences in length between the original target genes and the gene disruption construct that replaced them revealed which samples were transformants, which were wild-type DK80 and which were heterokaryons, containing both the intact and knockout genomes. Because of *Cp*'s propensity for anastomosis, wild-type spores that germinated in the regeneration medium fused with knockout spores. No mononkaryon mutant colonies were expected or observed at this stage.

PDA and PDA+hyg plates containing heterokaryon colonies were incubated on the benchtop for up to 10 days to produce conidia. The heterokaryon colonies generally did not produce fruiting bodies on PDA+hyg medium, so spores in most cases had to be harvested from colonies grown on PDA. Spores were collected by pipetting 10 µl drops of sterile distilled water onto ripe fruiting bodies to allow spores to disperse into the water droplet, then drawing the droplet back into the pipette and transferring it to to a 1.5 µl tubes containing 1 mL of distilled water for dilution (figure 3). Serial dilutions of suspension were transferred to fresh +hyg plates and spread over the surface with sterilized a glass spreader. Any mycelia that grew on these selective medium plates were subsequently transferred to fresh +hyg plates, allowed to grow out until DNA could again be extracted, and analyzed by PCR to identify true monokaryon knockout cultures.

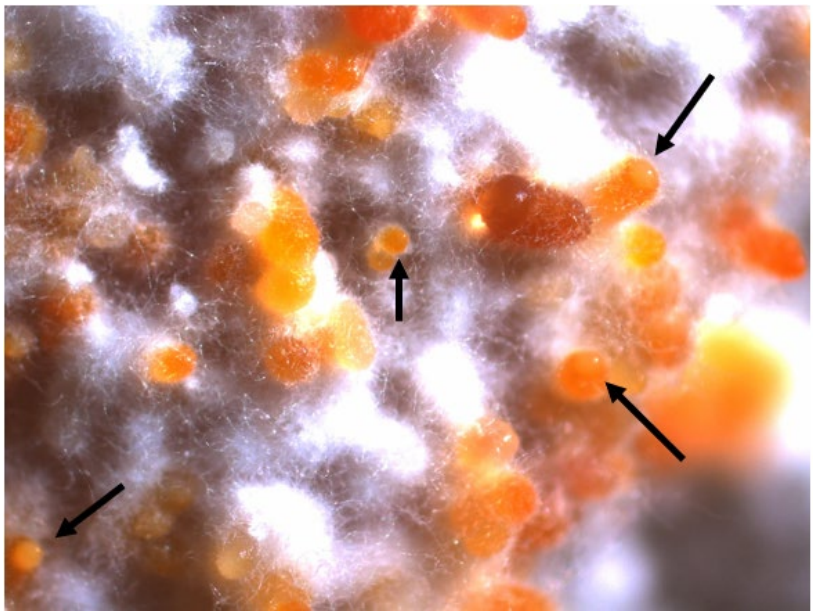


Figure 3. Ripe *Cp* fruiting bodies at 340x magnification. Black arrows indicate where pycnidia exude a water-soluble spore suspension that can be diluted and collected by pipette.

Examination of knockout strain phenotype

To observe phenotypic changes resulting from the deletion of target genes, nine 5 mm plugs of monokaryon mycelia for each knockout strain were transferred from the +hyg media, where they were grown and maintained, to three types of petri dishes containing different –hyg media: Endothia parastica complete (EPC) medium to observe growth under nutrient-rich conditions, water-agar (WA) medium to observe growth under nutrient-poor conditions, and chestnut bark agar (CBA) medium to simulate the environment present in American chestnut bark. (Formulas for these media are provided in appendix I.) There were three replications for each type of medium.

Mycelial diameters were measured daily with a ruler to chart the growth of the fungal strains, and observations made concerning the appearance of pigment and fruiting bodies. Photographs containing one plate each, representing the medium mycelial diameter for each medium type, were taken at regular intervals.

Because diameter increases faster for mycelia in nutrient-restrained conditions (Jennings and Lysek, pg. 13), diameter can be a poor indication of biomass accumulation. To measure relative biomass accumulation, nine additional plugs of knockout strain and nine of untransformed DK80 mycelia were made on plates with cellophane layers placed over the three types of medium. The cellophane was pre-cut to have a uniform dry weight prior to inoculation, and was peeled off with the

mycelium at seven dpi, blotted with sterile filter paper to remove liquid that occasionally accumulates between the mycelium and the cellophane (this step was only necessary for CBA medium), and weighed in wet conditions (weight when peeled off the medium) and dry conditions (after the mycelium-laden cellophane was allowed to dry at least 24 hours in a 37°C incubator. The average weight of the non-inoculated cellophane controls was subtracted from the average weight for each mycelium type, and the results used to compare the relative accumulation of biomass by the knockout strain versus wild type DK80, using a Student's *t*-test (α=0.05).

Examination of knockout strain virulence in planta

While *in vitro* observations can reveal some differences between the wild type and knockout strains, there may be other relevant environmental factors in the live bark of chestnut trees that can influence fungal growth and development. In addition, inoculations into live American chestnut tissue are necessary to observe any effect of the gene knockouts on the virulence of *Cp*. Inoculations into live mature American chestnut trees was not feasible due to the lack of available non-blighted trees, and due to restrictions on the release of genetically modified pathogens into the environment. Therefore two other types of *in planta* assays were used to observe fungal growth and development in American chestnut tissue: a detached stem assay widely used in *Cp* research (Elliston 1978, Jacob-wilk *et al* 2009, Levine-Double personal communication), and a small stem assay developed in cooperation between the author and the science staff of the American Chestnut Foundation (Saielli and Levine 2019).

The detached stem assay

For the detached stem assay, non-blighted branches 4-8 cm in diameter and 0.5 – 1.0 meters in length were harvested from dormant American chestnut trees in orchards maintained by the Maryland Chapter of the American Chestnut Foundation in January 2019. The ends of the branch segments were sealed with melted food grade wax to preserve moisture and put in a -20 freezer for storage until use. When removed for inoculation, stems were left to thaw to room temperature, bathed in a 10% dilution of household bleach for 15 minutes, then allowed to dry on newspaper. Inoculations sites were chosen at points along the stems that were away from branches or damaged bark, and at least 10 cm apart from each other. Sites were marked with a randomly assigned site number with typing correction fluid. Five mm plugs of inoculum grown on PDA were placed, mycelium side in, into holes drilled through the bark of the stems with an ethanol-sterilized 3/8 inch steel punch in a cordless drill. Inoculum was sealed in place with segments of masking tape. Inoculated stems were placed in sealed, translucent plastic boxes in a greenhouse at 25°C, with approximately 70% humidity and a natural daylight cycle. The length and width of resulting *Cp* cankers were measured weekly, and estimates of a normalized canker length (NCL) were made. NCL is the square root of the area of an ellipse calculated using the actual length and width of the canker (figure 4). The NCL is a linear measurement that allows us to compensate for differences in the ratio of canker length to canker width that results from variance in diameter between stems.

$$NCL = \sqrt{\frac{\pi lw}{4}}$$

Figure 4. Formula for normalized canker length, where l = observed canker length and w = observed canker width.

Observations were noted concerning the emergence and phenotype of fruiting bodies. Fifteen replicates for each knockout strain and for the inoculations with DK80 were randomly distributed among the available inoculation sites, along with 15 inoculations with sterile agar medium as negative controls, 15 of the virulent *Cp* strain EP155 as positive controls and 15 of the confirmed weakly virulent *Cp* strain strain SG2,3. EP155 and SG2,3 cultures were obtained from the American Chestnut Foundation under APHIS license. Failed inoculations, or cankers affected by obvious contamination by naturally occurring fungi were deleted from the data set. The mean NCL for each strain and observations concerning the emergence of fruiting bodies were used as proxy measures for fungal virulence. Differences in NCL between knockout strains and the DK80 parent strain were calculated using Student's *t*-test (α=0.05).

The small stem assay

Detached stem assays have been shown to be good predictors of canker size resulting from inoculation in live trees (Elliston 1978, Jacob-Wilk *et al* 2009). The cambium layer of stem segments preserved in this manner is made up of live cells with intact constitutive and induced defense capabilities. The assay can be completed within one month and carried out at any time of year using frozen material. However, the detached stem assay may be limited by the inability of live cells to draw on distal

resources as they might in a live tree. This is the reason a second type of virulence assay was conducted in the bark of small seedlings in their first year of growth, the small stem assay.

For the small stem assay, pure American chestnut seeds were obtained from a single pure American parent tree by the Maryland Chapter of the American Chestnut Foundation. These seeds were stratified at 4°C and planted in a greenhouse in late 2017, and again in late 2018. Inoculations were carried out when enough seedlings had stems 6 mm or greater in diameter at the base. Inoculations involved placement of plugs (approximately 1mm x 1 mmx 5 mm) of knockout strain or DK80 mycelia grown on PDA medium into 1 x 5 mm incisions made through the bark, but not into the heartwood of the stems, with one inoculation per stem. The incision was made with a 2 mm cork borer cut at a 45° angle and sharpened with a scalpel. At least five incisions were inoculated with sterile agar medium as negative controls. All tools were sterilized with ethanol and flared between contact with different fungal strains. After placement of the mycelial plugs into the incisions, each inoculation site was wrapped tightly with a 2 cm wide piece of pre-stretched parafilm and twisted in place. The parafilm was left in place for seven days, and measurements of the length of resulting cankers were taken at 14-day intervals. At each measurement, the length of each canker was recorded, and the trees were also assigned a qualitative score, using the decision tree in figure 5. The qualitative score was designed to characterize stages of canker development, and to be used to compare the rate of canker development between weakly virulent strains that do not result in mortality.

Is the stem distal to the inoculation site dead?
If yes, **Score=5**
If no, is there a canker that is sunken in the middle compared to the margins, or which has *Cp* fruiting bodies?
If yes to either, **Score=4**
If no, does the canker fully encircle the stem, or does it extend more than 5 mm from either end of the inoculation site?
If yes to either**, Score=3**
If no, is there any obvious sign of fungal infection around the inoculation site?
If yes, **Score=2**
If no, **Score=1**

Figure 5. Decision tree for qualitative rankings of small stem assay cankers.

Measurements continued for 14 weeks. At the end of the period, mean canker length and the mean number of weeks until stem death were calculated for each fungal strain. Comparisons of canker length, mean days of stem survival (days until the portion of the stem above the inoculation site dies) and qualitative scores (if necessary) were to be made in Excel using *t*-tests with unequal variance for pairwise comparisons between any given knockout strain and DK80 and negative controls, or by ANOVA if testing multiple knockout strains in one assay.

Results and Discussion

Profiles of target genes

A comparison between the *Cp* strain EP-155 and *Gc* strain UCSC1 reference genomes, using the gene selection strategy described in chapter 2, yielded 33 predicted homologous genes with E-values less than 10^{-6} . Of these, three had been reported to be essential genes (lethal when impaired) in the model ascomycete yeast

Sc. Of the remaining 30, six had previously been shown to be up-regulated at least 2-fold in *Gc* (UCSC1) haustoria, the feeding structures of the pathogen (Wu *et al* 2018). None of these six genes (*TG1-TG6*) have previously been characterized in *Cp* or *Gc*, and a literature search revealed no reference to the genes in studies of either species. However, BlastP searches of the predicted proteins in *Cp* produced over 100 homologues for each, and homologues of each of the genes have been characterized or studied in *Sc* (table 4).

Table 4. *Cp* Target Genes 1 through 6 and their homologues in *Gc* and *Sc*.

Target Gene	*Cp* strain EP-155 protein ID [1]	*Cp* gene length	*Cp* amino acid sequence length	*Gc* strain UCSC1 protein ID number[2]	*S. cerevisiae* homologous protein/gene[3]
TG1	96843	2136	564	32023	YCR068W/*ATG15*
TG2	355196	1003	110	210066	YDR382W/*RPP2B*
TG3	242884	926	286	78010	YJL158C/*CIS3*
TG4	347494	1086	234	120011	YBR171W/*SEC66*
TG5	320126	1569	302	132010	YKL120W/*OAC1*
TG6	334581	709	197	197034	YDL046W/*NPC2*

[1] From U.S. Department of Energy Joint Genome Institute (JGI) genome.jgi.doe.gov/Crypa2, [2] From Genbank accession number MCR00000000.1, [3] from www.yeastgenome.org.

Though all six target genes were predicted by analysis with SignalP v. 3.0 to encode proteins with an N-terminal signal peptide, and analysis with TMHMM did not predict transmembrane domains in any of the six, an examination of the most similar proteins discovered by BlastP search suggested that *TG1, TG2, TG4* and *TG5* encode proteins that are predicted to be targeted to intracellular membrane compartments, and that the TG3 protein may be a components of the cell wall. A BlastP search of

TG6 homologues provided no clues regarding the subcellular localization of these proteins, suggesting it may be a secreted effector protein.

Barakat *et al* (2009 and 2012) examined the transcriptome of *Cp*-infected and non-infected Chinese and American chestnut. We accessed the cDNA reads from these studies (available at https://www.hardwoodgenomics.org) and used Hisat2 to map the reads to the *Cp* genome. However, only 2,924 reads out of a total of 129,508 mapped to the *Cp* genome rather than to the chestnut genome. There were five reads corresponding to *TG2*, and none corresponding to any of the others. We believe the RNA extraction methods used by Barakat *et al* must have been optimized for plant tissue, which was the subject of their study.

We also examined two other studies that looked at the differential expression of *Cp* genes. Kim *et al* (2012) did a proteomic analysis of *Cp*, comparing growth of an uninfected strain and an isogenic hypovirulent (infected with hypovirus CHV1) in PDA medium and PDA amended with tannic acid, which has been shown to induce expression of certain pathogenesis-related genes in *Cp*. Wang *et al* (2016) also studied the influence of hypovirus on gene expression in *Cp*. Neither identified any of our target genes among the genes up- or down-regulated under the conditions of their respective studies.

TG1: a putative autophagy-related protein

A BlastP search of the predicted protein from the *Cp TG1* gene generated 92 non-duplicate hits from the NCBI non-redundant protein sequences database, all corresponding to fungi, and with an average amino acid sequence identity of 76%. Seventy percent of the TG1 hits were in plant-pathogenic fungi, with the closest homologues found in fungi of the Sordariomycete class, which also includes *Cp*, within the phylum Ascomycotina. TG1 homologues are generally annotated as "predicted lipases, or autophagy lipase protein Atg15." While we have found no reference in available literature to research on the *TG1* gene in *Cp* or any powdery mildew species, its homologue in *Sc* has been well-studied.

Atg15 is one of several highly-conserved autophagy related protein genes found in eukaryotic cells. Autophagy is a process by which damaged or unnecessary cytoplasmic components and toxic aggregates can be degraded within the vacuole (in fungi and plants) or lysosome (in animals) and recycled (Delorme-Axford *et al* 2018). Atg proteins mediate a process by which specialized structures capture target substrates and deliver them to the vacuole/lysosome, where they are broken down into raw materials that can be exported back to the cytosol for re-use (Epple *et al* 2001). Autophagy is essential for cell growth and development and occurs at a low level in all cells. It increases significantly during nutrient starvation, pathogen infection or other stress conditions, and helps maintain homeostasis (Delorme-Axford 2018, Ramya *et al* 2016).

Epple (2001) reports that the *Sc ATG15* gene is essential for the breakdown of autophagic bodies in the vacuole. Ramya *et al* (2016) describe Atg15 as the only lipase among Atg proteins in *Sc* and report that it preferentially hydrolyses the cellular membrane component phosphatidyl serine. Parzych *et al* (2018) report that one role of Atg15 in the vacuole is to break down liquid droplets, specialized organelles that can store neutral lipids and sequester toxic compounds such as fatty. They further report that yeast cells lacking the Atg15 protein do not entirely lose the ability to break down lipids through autophagy, but that such cells lose viability in nitrogen starvation conditions within six days, sooner than is the case for wild-type cells (Parzych *et al* 2018).

While the exact roles of *TG1* in *Gc* and *Cp* are not known, the studies of *Sc* discussed above suggest that it may help plant-pathogenic fungal cells cope with the nutrient-poor conditions that exist within host tissue by recycling nutrients. Also, in the case of *Cp*, fatty acid molecules produced by host plants have been shown to inhibit fungal growth (Samann *et al* 1978), and *TG1* may form part of the fungal pathway that sequesters, traffics and breaks down these anti-fungal compounds, among other lipids.

TG2: a putative 60s ribosomal subunit P2 acidic protein

A BlastP search of the predicted protein from the *Cp TG2* gene generated 91 non-duplicate hits from the NCBI database, all corresponding to fungi, and with an average amino acid sequence identity of 76%. The closest homologues of the TG2 protein were reported in other ascomycete fungi of the Pezizomycete subphylum, to

which *Cp* also belongs. Compared to the other target genes in this study, *TG2* homologues were less concentrated among plant pathogens, with a greater percentage of hits corresponding to saprophytes or animal/insect pathogens. The high amino acid sequence conservation of the TG2 protein in fungi of various classes and lifestyles suggests that the protein is ancient, and that it likely plays an important housekeeping or regulatory function. BlastP hits were generally annotated as "putative 60s ribosomal subunit proteins," or "P2 acidic proteins." A literature search produced no previous research into the *TG2* gene in powdery mildew or *Cp*, but the gene has been the subject of several studies in *Sc*, in which it is characterized as a P2 acidic protein.

In Eukaryotic species, P1 and P2 acidic proteins interact with the P0 protein to form the ribosomal stalk, a structure which is involved in translation elongation (Remacha 1995). Remacha reports that there are genes encoding two forms each of the P1 and P2 acidic proteins in *Sc*, and an analysis of P1/P2 mutants suggested that their absence affected the rate of cell growth, but not cell viability. The absence of different P1/P2 proteins from the ribosome did not affect expression of different metabolic pathways in the same way, and Remacha (1995) hypothesized that the different acidic proteins play different roles in the translation of different mRNAs. Cardenas (2019), studying P1/P2 mutants in *Sc* found the absence of certain acidic proteins affects the translation of specific mRNAs, and to leads to certain phenotypic traits, such as cold-sensitivity. Cardenas describes P1 and P2 proteins as part of a stalk assembly mechanism that can produce heterogeneous ribosomal stalks.

Cellular expression of various proportions of P1 and P2 proteins appears to function as a regulatory mechanism moderating the relative efficiency of translation of different mRNAs (Cardenas, 2019). Such a regulatory mechanism may play a role in enabling fungi to adapt to changing environmental conditions associated with changes in internal or environmental conditions at different points in their lifecycles.

TG3: a putative cell wall mannoprotein

The annotation of the *TG3* gene changed significantly between the first and second versions of the *Cp* genome published. A reannotation of the *Cp* genome was performed at the University of Southern Mississippi in 2017, and included a third annotation for the *TG3* gene, but has not yet published (Levine-Dawe personal communication). BlastP searches were carried out on the proteins predicted by all three annotations, and all three produced a highly similar set of hits from the NCBI non-redundant proteins database. After removing duplicate species, we obtained a list of 96 fungal proteins with an average amino acid sequence homology of 52%. Of these, 75 were found in plant, animal or insect pathogens. Hits with the highest similarity scores were all plant pathogens in the fungal order Diaporthales, to which *Cp* also belongs. Annotations of the putative homologues included "covalently linked cell wall protein," "cell wall mannoprotein," "cell wall Cis3 Protein," "Pir3 protein," and "Pir5 protein." All of these refer to a family of glycosylated proteins found in the outer cell walls of certain fungi. (Hsu *et al* 2015, Klis *et al* 2006, DeGroot *et al* 2005). *TG3* is the only member of this protein family that has been annotated as such in *Cp*.

A literature search found no previous reference to the TG3 gene, or any genes bearing the same annotations, for *Cp* or *Gc*, but there has been extensive research into the localization and possible roles for such glycoproteins in *Sc*, and some discussion of homologues in filamentous fungi, especially ascomycetes (DeGroot *et al* 2005). The PIR (proteins with internal repeats) family consists of mannose-containing glycoprotein constituents of the fungal cell wall. DeGroot *et al* (2005) describe the cell walls of fungi from which such proteins have been isolated as consisting of an internal skeleton of 1-3 beta-glucan chains, surrounded by a denser layer rich in proteins. DeGroot and others speculate that the repeats in PIR proteins allow them to bind to multiple 1-3 beta-glucan molecules and stabilize the otherwise highly flexible and porous cell wall (Hsu *et al* 2015, Klis *et al* 2006, DeGroot *et al* 2005.) PIR proteins appear to have more than a passive reinforcement function, however. The incorporation of PIR proteins and other proteins into the cell wall is tightly regulated, based on location and on what stage of the cell cycle the cell is in. Cell wall protein composition is also influenced by osmotic pressure and physical stress/damage and other environmental conditions, through a variety of signaling pathways (Hsu *et al* 2015).

Possible functions for PIR proteins enumerated by DeGroot *et al* (2015) include: water retention, maintaining cell wall integrity in response to stress and/or growth and development, adhesion to the host and protection from host defenses. It has been observed that yeast cells lacking multiple PIR proteins swell, and grow slowly. The absence of individual PIR proteins have been shown in yeast to increase sensitivity to

plant antifungal defensive chemicals such as osmotin, and antibiotics such as hygromycin (DeGroot *et al* 2015).

For both *Cp* and powdery mildew species, *TG3* may help the fungi adapt to live inside living host tissue by strengthening the cell walls of invasive structures and protecting the fungi from host defensive proteins. It is also possible that the TG3 mannoprotein may migrate into host tissues and play a role there, in which case it would be a *bona fide* secreted effector protein. Early studies of mannoproteins in *Sc* found that about 5% of mannonprotein migrated into the growth medium, and that this happened at a constant rate throughout the cell cycle. The researchers believed that the released mannoprotein was either synthesized de-novo or represented mannorproteins that were non-structural in nature (Kratky *et al* 1975).

TG4: a putative pre-protein translocase

A BlastP search for homologues of the TG4 protein produced 96 non-duplicate fungal hits with an average amino acid sequence identity of 70%. Homologous proteins in other plant pathogens in the fungal order Diaporthales were especially highly-conserved, with identities over 80%. Analysis of the TG4 sequence with TMHMM 2.0 suggested one transmembrane domain located within the first 30 amino acid residues of the protein, which we initially discounted as coinciding with the N-terminal signal peptide. However, annotations of TG4 homologues, where provided, consistently referred to the Sec66 translocase, a subunit of the Sec62/63 translocation complex, which is an integral membrane protein of the endoplasmic reticulum. A literature search found no references to the *TG4* being previously studied in *Cp* or

powdery mildew, but the protein was extensively examined in *Sc*, and its possible role and functions have been reported in some other yeasts and filamentous fungi. The Sec66 protein , also known in *Sc* as Sec71 and Kar7, interacts in an auxiliary manner with the Sec62/63 complex, membrane proteins which, in turn, interact with the Sec61 pore in the endoplasmic reticulum (ER) membrane. It is part of a heteromer associated with post-translational translocation, especially of secreted proteins (Rapaport 2007). The role of TG4 may not be entirely restricted to secreted proteins, however. Jung *et al* (2014) report evidence that the Sec66 protein also helps regulate topogenesis of membrane proteins in eukaryotic cells.

The Sec66 protein is not essential for yeast cell growth or survival (Feldheim 1993). *Sc sec66*-null mutants were found to be viable at 30°C but not at 37°C (Feldheim 1993) but the role of the protein is clearly more than just to stabilize translocation functions at high temperatures. *Sec66* has been shown to be important in several disparate cellular functions in yeast, and in other fungi. For example, Nishikawa *et al* (2008) found that *sec66*-null mutants were unable to accomplish the karyogamy associated with sexual reproduction in yeast due a failure of outer nuclear envelopes to fuse. Katta *et al* (2015) found that the absence of a functioning *Sec66* gene led to defects in spindle pole body duplication during mitosis in yeast. Both Nishikawa and Katta noted that the defects they observed occurred at moderate temperatures (30°C) as well as at high temperatures (37°C). Lee and Heitman (2012) reported that sec66 was necessary for the completion of opposite sex and unisex mating in the dimorphous yeast *Cryptococcus neoformans*. Kang and Jiang (2005) found *Sec66* to

be one of several protein secretion-related genes upregulated during the transition to filamentous growth in dimorphic yeast. It is notable that all of these cellular functions are, in turn, induced by chemical (e.g. mating pheromones) or environmental (temperature, nutrient deprivation) signals.

Whether *SEC66* affects the various cellular functions described above through its role as a translocator of other proteins, or in a manner entirely separate from its translocation function remains unclear. What the examples provided in the literature have in common, however, is that *TG4* homologues come into play when fungal cells are experiencing environmental stress or developmental change, suggesting that this otherwise dispensable protein may be an important regulator of cellular responses necessary to cope with such changes.

TG5: a putative mitochondrial carrier protein

A BlastP search of the TG5 protein revealed strong amino acid sequence identity among homologues in the highest scoring fungal hits. Sequence identity averaged 84% with coverage ranging from 95 to 100%. The closest homologues to TG5 were found in other plant pathogenic fungi of the order Diaporthales, to which *Cp* also belongs. Numerous animal and insect pathogens were represented, but relatively few saprophytes. Most hits were annotated as "mitochondrial carrier protein," "mitochondrial inner membrane protein," or "mitochondrial oxaloacetate carrier protein." A literature search found no previous examination of the TG5 gene in *Cp* or any powdery mildew species. The characterization of the protein as a mitochondrial carrier is based on the discovery of a homologous gene, *OAC1* in *Sc*.

The *Sc* gene OAC1 has been localized to the mitochondrial inner membrane (Palmieri 1999). Analysis of the TG5 protein with TMHMM 2.0 initially predicted no transmembrane domains, but a graphical analysis shows up to five predicted transmembrane domains with varying degrees of probability. Annotations of the *Sc OAC1* gene report three transmembrane domains that correspond to repetitions of the carrier protein sequence.

OAC1 has been shown to transport oxaloacetate, sulfate and malonate into mitochondria, and to transport α-isopropylmalate (IPM) from the mitochondria to the cytosol. IPM is used in leucine biosynthesis (Palmieri 2016). There is some debate in the literature about the relative importance of transport into versus out of mitochondria, given that other pathways exist in *Sc* for transporting these compounds in each direction.

The *OAC1* gene in *Sc* is not essential for growth or survival. Yeast cells lacking *OAC1* showed a slightly reduced growth, due to partial auxotrophy for leucine, which was correctable by the complementation of the *OAC1* knockout with a plasmid carrying the *OAC1* gene or by growing the yeast in media containing leucine (Marrobio 2008). The *OAC1* gene's expression is downregulated by and inhibited by α-ketoisocaproate (KIC), a precursor and metabolite of leucine produced by the mitochondria (Marrobio 2008). How these regulatory relationships are connected is not clear, but *OAC1* does appear to be subject to an overarching regulatory mechanism. The fact that the *TG5* homologue in powdery mildew is upregulated in

haustoria, and the fact that *OAC1* supplies raw materials for leucine biosynthesis when leucine is scarce have a common thread--in both cases the TG5 homologues play a role in helping cells adapt to changes in the availability of nitrogen.

TG6: a possible effector protein with an ML domain

A BlastP search for homologues of the TG6 protein produced 83 non-duplicate hits in fungi with an average amino acid sequence identity of 27%, far lower than any of the other five target genes. Like *TG1* (at 70%), most *TG6* hits (60%) were among plant pathogenic fungi. Annotations of homologous proteins included "ML-domain containing protein" and "phosphatidylglycerol /phosphatidylinositol transfer protein (PG/PI-TP)."

ML-proteins (MD-2-related lipid recognition proteins) were first characterized by Inohara and Nunez in 2002, who described them as "single-domain proteins predicted to form a β-rich fold containing multiple strands, and to mediate diverse biological functions through interacting with specific lipids." The highest scoring TG6 hit was a putative PG/PI-TP transfer protein in *Gc*, but the *Cp* TG6 protein only shared 28% amino acid sequence identity with this protein. A BlastP search beginning at residue 65 of the *Cp* TG6 gene, the point where the predicted ML-domain begins, produced a nearly identical set of hits to the whole protein. A separate BlastP search for the first 65 amino acid residues (without the ML-domain) produced only one significant hit, a deltaproteobacterium found in marine sediment with 35% sequence identity. This appears unrelated to TG6.

ML proteins are found in numerous animal, plant and fungal genomes. They show no sequence homology to non-specific lipid interacting proteins, leading Inohara and Nunez (2002) to the hypothesis that they interact only with specific lipids with a diverse range of biological functions. Subsequent research has produced results consistent with this hypothesis. For example, the TG6 homologue in *Aspergillis oryzae*, previously characterized as a membrane-targeted lipid transfer protein with a specific affinity for phosphatidylglycerol /phosphatidylinositol, is an ML-protein of unknown function. In animals, the MD-1 and MD-2 proteins, from which the ML-domain was first characterized, are co-factors with Toll-like receptors in lipopolysaccharide signaling-based anti-bacterial immune responses (Inohara and Nunez, 2002). Berger *et al* (2005) observed a strong homology between the *Sc* gene NPC2 and the human hNPC2 gene, defects of which are implicated in the hereditary lipid storage/cholesterol metabolism disorder Niemann-Pick disease type C. Berger *et al* (2005) were able to restore normal cholesterol transport in hNPC2-null mutant animal cells by complementing them with NPC2 from yeast, and speculated that the gene's function in yeast is to maintain lipid homeostasis.

Menardo *et al* found six ML proteins among suspected effector proteins produced by the barley PM fungus *Blumeria graminis* (Menardo *et al* 2017). Research on arbuscular mychorrizal (Zeng *et al* 2006) and ectomycorrhizal (Sebastiana, 2017) fungi suggests that ML proteins play a role in lipid signaling in the host-symbiont interaction.

Information available about *Cp TG6* is not sufficient to suggest where this protein localizes in the cell after entering the ER lumen, or whether it is secreted, or whether it interacts with cellular or extracellular lipids. However, *TG6* appears to be the most likely of the six target genes that may encode an effector protein. With less than 300 amino acid residues, it meets criteria commonly used to screen for candidate effector proteins, including short protein length, and lack of close homologues or homology to proteins with known functions (Kim *et al* 2016, Sperschneider 2018). With respect to cysteine-richness, the TG6 protein is at the extreme low end (2%) of the 2%-20% range considered typical of fungal effector proteins (Lu and Edwards 2016). When we analyzed each of the target gene proteins using EffectorP v2.0 (effectorp.csiro.au/software.html), a machine learning-based platform for predicting effector proteins (Sperschneider 2018), it gave TG6 a 0.828 probability of being an effector protein. TG4 received a 0.531 probability, but its homologues are thought to be integral proteins of the ER. The other target gene proteins were assigned probabilities close to zero.

The Generation and Characterization of Knockout Strains

Multiple attempts at transformation

Transformation was carried out on three occasions, in February 2018 (*TG1-TG4* only), August 2018 (all six target genes) and November 2018 (all six target genes). At each attempt at transformation, one sample of spheroplasts was also put through

the transformation process without any gene disruption constructs as a positive control, in each case confirming that the spheroplasts were viable.

During the February 2018 round of transformations, we were able to recover heterokaryon mycelia for each attempted knockout. These could grow on PDA + hyg medium, but development of fruiting bodies was very delayed. Each isolate was also grown on plain PDA until it produced oozing fruiting bodies, and the spores were collected in droplets of water with a 10 µl pipette. Ten µl of spore suspension was diluted in 1 mL of distilled water, and serial dilutions, ranging from 2 µl to 200 µl of this suspension were spread on PDA+hyg medium. We were only able to recover monokaryon knockout colonies for the *TG4* knockout. Spores from *TG1*, *TG2*, and *TG3* heterokaryon strains did not germinate on PDA+hyg medium. We attempted hyphal tip cultures for *TG1*, *TG2* and *TG3* in case spore germination was suppressed more than vegetative growth by the hygromycin. Heterokaryon mycelia from these isolates were grown in plates with WA+hyg medium, where they formed sparse and branchy mycelia, from which we excised hyphal tips under a dissecting scope and transferred them to PDA+hyg plates. While some of these hyphal tip cultures subsequently grew well, PCR visualization of the DNA segments corresponding to the target genes showed that all of the hyphal tip cultures that survived on the PDA+hyg plates were still heterokaryons. We found it not feasible with the equipment available to cut hyphal tips that did not contain multiple nuclei. The failure to generate monokaryon colonies by either single spore or hyphal tip cultures raised the question of whether the deleted wild-type genes were essential.

However, we also noted that the TG4 knockout was the only strain to be developed during the first transformation with a final PCR-amplification step. The *TG1-TG3* knockouts were made with non-linearized plasmid DNA. When the transformation and regeneration process was repeated in August 2018, we added a final PCR amplification step for all gene disruption constructs to ensure that all of the DNA used in the transformation was linearized.

The August round of transformations also added *TG5* and *TG6*, for which gene disruption constructs had not been available in February. We also used a lower dosage of hygromycin (30 mg/mL instead of the 50 mg/mL recommended by Churchill *et al*) in the regeneration and selection media, in case the failure of *TG1-TG3* spores to germinate was due to a naturally lower expression of those genes compared to *TG4*, resulting in less production of the hph enzyme. However, at 30 mg/mL, we observed that untransformed mycelia could survive and grow on solid (but not in liquid) media, making it difficult to screen out wild-type colonies. We obtained heterokaryon colonies for TG1 and TG6 at this dosage, as confirmed by PCR measurement of DNA fragments corresponding to the target genes, but we could not isolate monokaryon colonies from single spore inoculation of PDA or EPC medium amended with 30 mg/mL of hygromycin.

The third round of transformations, beginning in November 2018, repeated the transformation and regeneration process for *TG1, TG2, TG3, TG5* and *TG6*, at a hygromycin dosage of 40 mg/mL of hygromycin, based on the previous successful

practices of other researchers (Levine-Zhang personal communication). However, we continued to observe untransformed, wild-type colonies growing on selection medium.

Use of a novel growth medium to induce spore germination

The failure of spores from the *TG1, TG2, TG3* and *TG5* knockout strains to germinate on solid media at any hygromycin dosage, including dosages on which heterokaryon and wild-type mycelia could grow, was puzzling. We did not consider it likely that all of these genes were essential for cell survival, as none of the homologues of the target genes were found to be essential in yeast, and one possible explanation may lie in what has been observed about how some of these genes are regulated in yeast. For example, the *ATG15* gene in yeast (the *TG1* homologue), was found to be up-regulated during starvation-induced autophagy (Delorme-Axford 2018). The *OAC1* gene (the *TG5* homologue) is down-regulated by leucine (Marrobio 2008), a downstream byproduct of the *OAC1* gene's activity. The *Gc* homologues of all six genes are also up-regulated in the haustoria of *Gc* (Wu *et al* 2018). All of this is consistent with the hypothesis that the target genes are induced by the conditions that prevail in live plant tissue, and enable the fungus to survive there.

The hygromycin resistance marker cassette we introduced includes its own constitutive fungal promoter, but some or all of the target gene loci may be subject to higher-level regulation, and the genes may only be expressed when induced by specific stimuli. For this reason, we decided to attempt a final round of single spore

inoculations, using heterokaryon cultures created in previous transformation rounds. Table 5 indicates the heterokaryon strains we had available. None were available for *TG5*. We collected asexual spores from these isolates, and spread them on two types of selection medium-- EPC medium and a chestnut induction medium (CIM)--each dosed with 50 mg/mL of hygromycin. CIM contains the trace ingredients and malt extract as EPC, but is amended with a water extract of American chestnut bark, uses sucrose in place of glucose to force the induction of digestive enzymes (Griffin 1994, pg. 135), and omits yeast extract to deprive the fungus of an exogenous source of nitrogen. (See Appendix I for the formula for CIM.)

Table 5. Heterokaryon and monokaryon isolates produced in attempts to knock out *TG1-TG6* by homologous gene replacement.

Target Gene	Heterokaryon isolates	Monokaryon isolates
TG1	1C, 1BB	None
TG2	2A	None
TG3	3A, 3C	None
TG4	4A, 4B, 4C, 4D	4A-5,4A-8,4B-1, 4B-2, 4B-3, 4B-4
TG5	None	None
TG6	6P, 6Q, 6R	None

In the final round of single spore inoculations, only spores from the TG6 knockout germinated and produced mycelia on EPC+Hyg50 medium, but fewer and more slowly than on CIM+Hyg50. The spores of all other knockout strains completely failed to germinate on EPC+Hyg50, but produced abundant mycelia on CIM+Hyg50. *TG1* knockout colonies grew more slowly than the other knockout strains, taking about twice as long to produce equivalent biomass. *TG3* knockouts were also

notable, producing significantly fewer single spore colonies than the other knockout strains.

PCR visualization of the target gene segments of isolates grown from spores that germinated on CIM, however, showed that they were all either heterokaryon (likely growing from hyphal fragments picked up with the spores) or wild-type colonies. A subsequent test found that untransformed DK80 could also grow on CIM+Hyg50, albeit somewhat impaired. Evidently some property or component of CIM neutralizes the effect of hygromycin.

At the end of three rounds of transformation, we were only successful in knocking out *TG4*.

Observations of the TG4 *knockout strain,* in vitro.

The *TG4* knockout strain generated four heterokaryon isolates, TG4A-D. Eight single spore colonies grown from isolates TG4A and TG4B were cultured on PDA+hyg40 medium, and all showed normal growth rates and high resistance to hygromycin.

Hyphae from eight of these single spore colonies, and from one sample of the wild-type EP155 strain of *Cp* (the wild-type parent strain of DK80) were transferred into separate flasks containing liquid EPC medium and allowed to incubate for several days. DNA was extracted, and used as template DNA in PCR reactions with *TG4*

flanking sequence primers. All *TG4* knockouts showed bands corresponding to the predicted longer length of the *TG4* gene disruption construct, while the EP155 sample showed a band corresponding the predicted length of the wild-type target gene plus flanking sequences (figure 6).

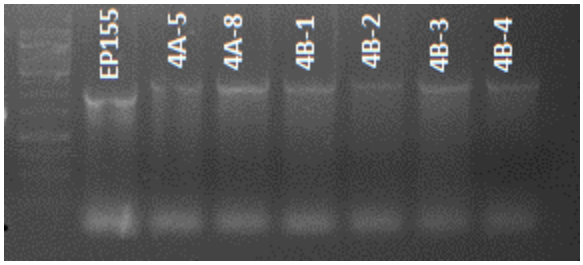


Figure 6. As predicted, the segment amplified between *TG4* flanking sequence primers is longer than the wild-type segment in isolate EP155 (identical to DK80).

Isolate dTG4A-8 ("d" indicates deletion) was chosen as a representative sample of the *TG4* knockout strain, and grown on EPC, CBA and WA media, as described in chapter 2. In vitro, dTG4A-8 exhibited consistently different growth rates and phenotype than DK80.

Radial growth measures were taken twice on CBA, EPC and WA media, once directly on media in March 2018, and once on cellophane over media in March 2019. The most notable difference between dTG4A-8 and DK80 was the former's relatively fast but sparse radial growth on synthetic media. Comparisons by Student's *t-test* on both occasions, each of which involved three replicates of each fungus type on all three types of media, showed that the diameter of dTG4A-8 mycelium was significantly larger (α=0.05) than that of DK80 on nutrient rich (EPC) and nutrient poor (WA) media (table 6), but not on CBA medium.

Table 6. *In vitro* comparison of DK80 and dTG4A-8 by radial growth and weight at final day of measurement (6-7 dpi). Bold indicates the isolate with greater measurement.

Medium	Diameter on medium (mm)			Diam. on cellophane (mm)			Mycelium weight (mg)		
	DK80	dTG4A-8	P-value	DK80	dTG4A-8	P-value	DK80	dTG4A-8	P-value
CBA	80.33	84.0	P=0.2756	**62.7**	58.5	**P=0.0247**	**10.0**	7.6	**P=0.007**
EPC	73.7	**81.3**	**P=0.0203**	57.**0**	**65.0**	**P=3.0x10^{-4}**	**107.3**	80.5	**P=8.5x10^{-5}**
WA	54.0	**60.7**	**P=5.0x10^{-4}**	46.5	**51.7**	**P=5.1x10^{-5}**	**2.8**	1.2	**P=0.026**

The rapid radial growth of dTG4A-8 appears to reflect poor fitness rather than vigor. Rapid sparse mycelial expansion is a normal response of filamentous to poor nutrition (Jennings and Lysek, 1999, pg. 13). The less vigorous growth of dTG4A-8 was substantiated by comparing the weights of dTG4A-8 and DK80 mycelia grown on layers of cellophane over the same three types of media, which showed that despite its rapid radial growth (figure 7), dTG4A-8 accumulated significantly less biomass than DK80 (figure 8). Notably, dTG4A-8 showed both less radial growth and less biomass accumulation than DK80 on CBA medium.

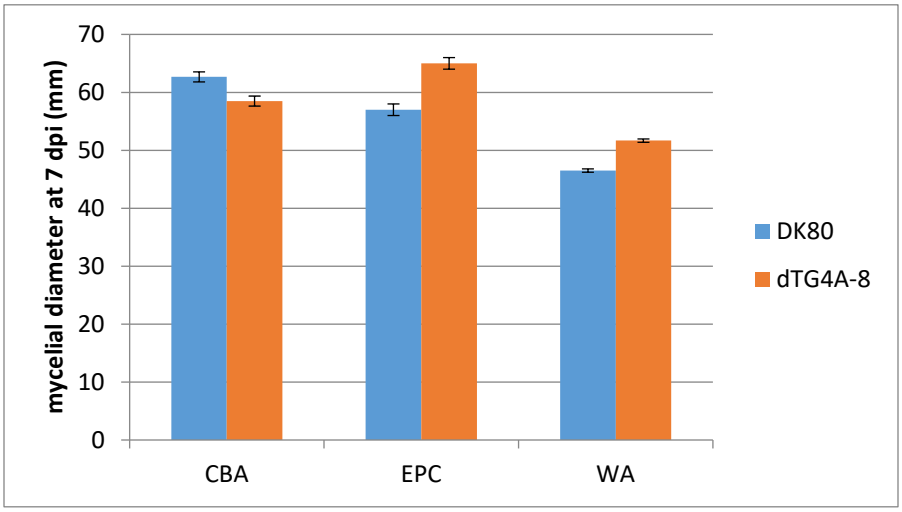


Figure 7. Comparison of mycelial diameter of DK80 and dTG4A-8 on three types of media, at seven dpi. Error bars indicate standard error.

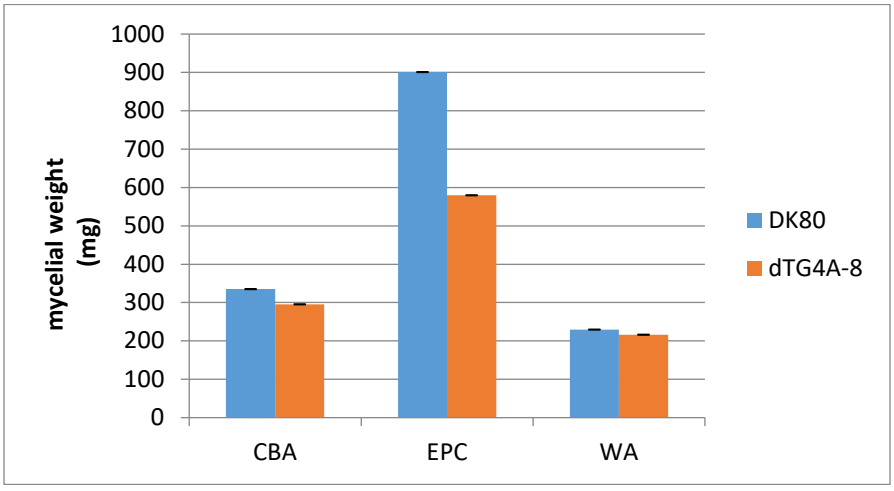


Figure 8. Comparison of accumulated weight of DK80 and dTG4A-8 mycelia grown on cellophane over three types of media, at seven dpi. Error bars indicate standard error.

In addition to different growth rates, there were also observable phenotypic differences between dTG4A-8 and DK80 (figure 9), including:

- dTG4A-8 reached each stage of development (e.g. appearance of pigment and fruiting bodies) later than DK80;
- dTG4 A-8 hyphae grew in disorderly, meandering manner compared to DK80 (figure 10);
- dTG4 A-8 produced less pigment than DK80 and the pigment was more tan/less orange than DK80 (figure 10);
- dTG4A-8 had fewer fruiting bodies, which appeared randomly within the mycelium, while DK80's more numerous fruiting bodies appeared mainly in concentric rings; fruiting bodies of both strains produced numerous, normal looking, viable conidiospores;

- dTG4A-8 showed no zonal growth, while zonal patterns corresponding to light cycle were distinct in DK80.

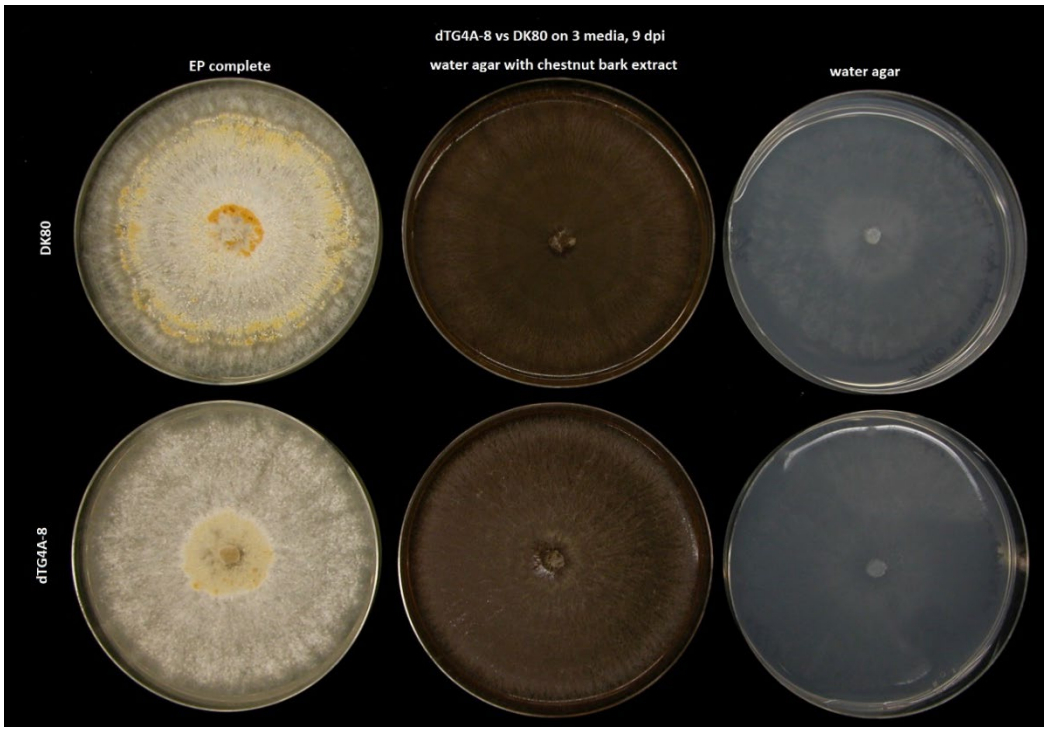


Figure 9. DK80 (upper row) and dTG4A-8 (lower row) on three media, left to right: EPC, CBA and WA.

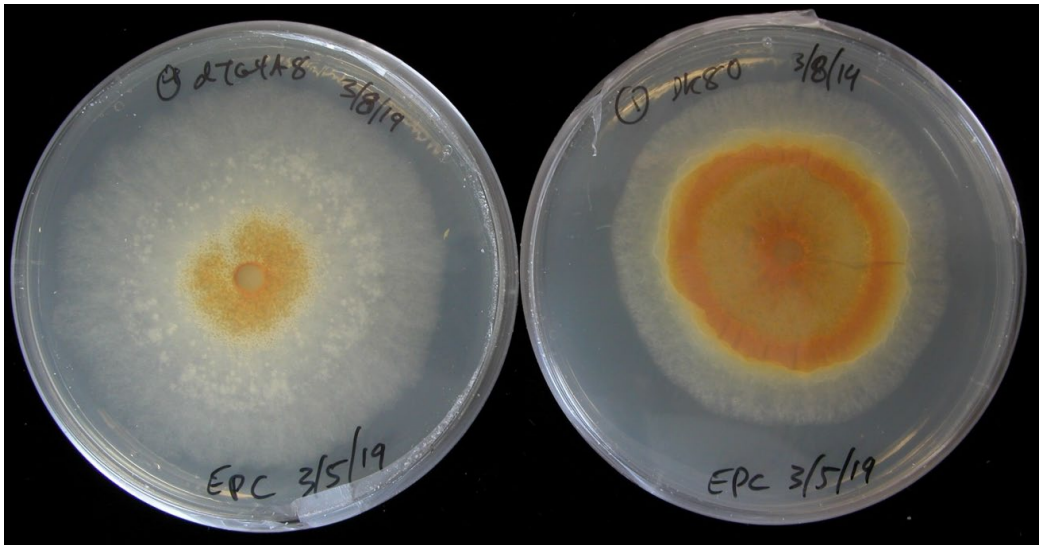


Figure 10. Side by side comparison of dTG4A-8 (left) and DK80 (right) inoculated on the same day, as seen from underneath shows that DK80 is denser, more pigmented and organized more distinctly into zones than dTG4A-8.

Light generally plays a role in regulating zonal growth and conidiation in filamentous fungi (Griffin 1994, pg. 345), so the difference between dTG4A-8 and DK80 in zonal growth may be due to a difference in light sensitivity. To test whether this was the case, we prepared six petri dishes with PDA medium, inoculated three with each *Cp* strain, and incubated them for eight days at room temperature in a dark box. When removed for observation, neither strain had yet developed fruiting bodies, which normally appear as early as day two in bench-top cultures. The darkness also abolished zonal growth in DK80, but dTG4A-8 grown in darkness appeared roughly identical to dTG4A-8 grown on the benchtop, with the exception of having almost no pigment (figure 10).

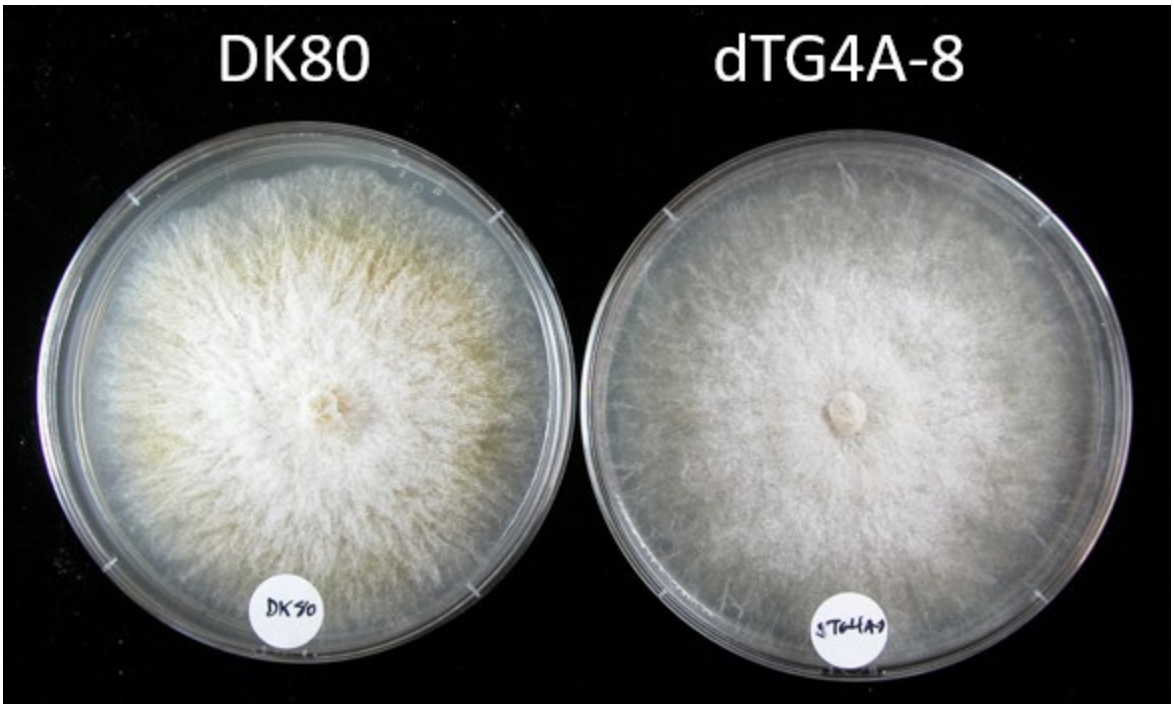


Figure 11. Growth in darkness abolished zonal growth in DK80 but did not change the morphology of dTG4A-8 mycelium.

Detached stem assay of the TG4 *knockout strain*

The dTG4A-8 strain was less virulent than DK80 against live American chestnut stem tissue in a detached stem assay conducted in March 2019. The assay used live, dormant American chestnut branch sections 2-5 cm in diameter, which were collected in January 2019 from orchards operated by the Maryland Chapter of the American Chestnut Foundation. Fifteen inoculations with 5 mm plugs of each of the following *Cp* strains, grown on PDA medium, were used in the assay:

- EP155: a standard virulent strain, used as a positive control;
- DK80: a virulent mutant derivative of EP155, and the parent strain of dTG4A-8;
- dTG4A-8: the *TG4* knockout strain produced in this research;
- SG2,3: a standard weakly virulent strain of *Cp*; and
- Sterile PDA: a negative control.

Cankers produced by dTG4A-8 were significantly smaller (P=0.03) at 24 days post-inoculation (dpi) than those produced by wild-type DK80. Figure 12 below shows photographs of DK80, dTG4A-8 and control inoculations on the same stem at 24 dpi. Figure 13, below, shows mean NCL for each type of inoculum. The *Cp* strains clustered into two groups, with the letter B representing the two statistically similar virulent strains (EP155 and DK80) and C representing the statistically similar weak strains (SG2,3 and dTG4A-8).

Figure 12. Photographs of DK80 (left), dTG4A-8 (center) and control inoculations (right) on the same stem, 24 dpi.

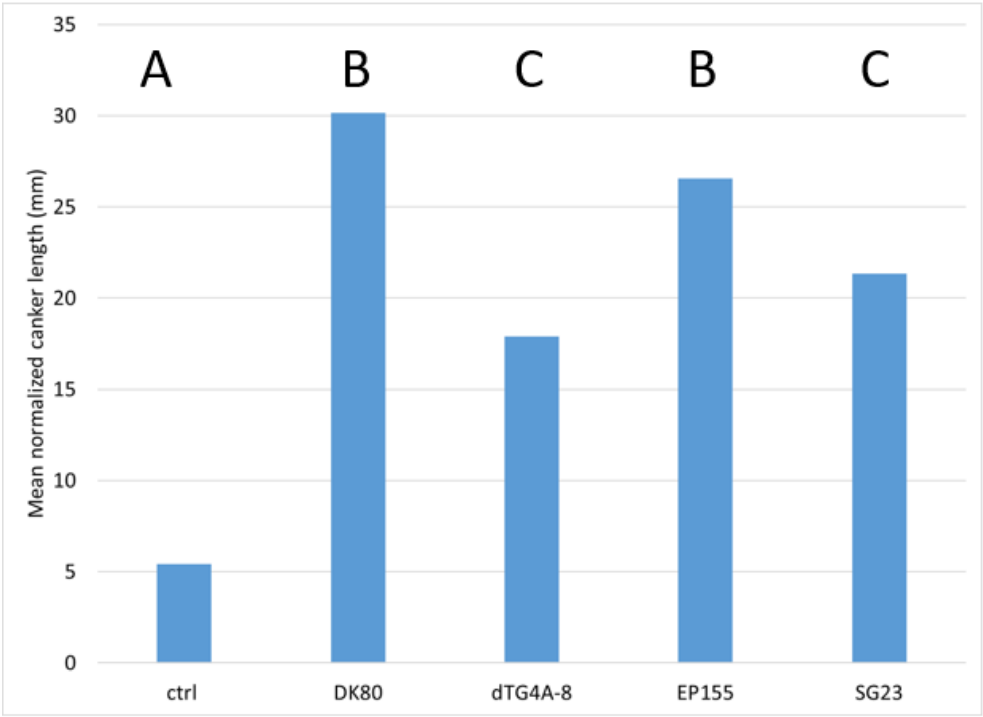


Figure 13. Mean normalized canker lengths for each type of inoculum used in the detached stem assay

Small stem assay of the TG4 *knockout strain*

A small stem assay was conducted in summer 2018 on 80 American first-year chestnut seedlings from the same mother tree, using fungal strains available at that time. These included dTG4A-8, and four heterokaryon knockout strains, TG1C(h),

TG2A(h), TG3C(h) and TG6P(h). DK80 was used as a positive control and sterile agar as a negative control. Unfortunately, the DK80 culture used for this inoculation had been sub-cultured too many times and had lost virulence. Only one out of 12 seedlings produced a canker. The small stem assay will be repeated with new seedlings and fresh inoculum in summer 2019. Nevertheless, data collected from the 2018 small stem assay produced meaningful results. The 11 seedlings inoculated with dTG4A-8 showed no stem mortality after 98 days of observation, the same result observed for the sterile agar control. All of the heterokaryon strains, which were inoculated into 10-12 seedlings each, produced mortality in at least three. Data for mean days of survival is shown in figure 14. In terms of canker length, dTGA-8 produced significantly) longer cankers (*t*-test, P=0.009) than the sterile control (the cankers of which were basically healed scars), but significantly shorter (*t*-test, $P=7x10^{-5}$) than TG3C(h), the strain that produced the next shortest mean canker length.

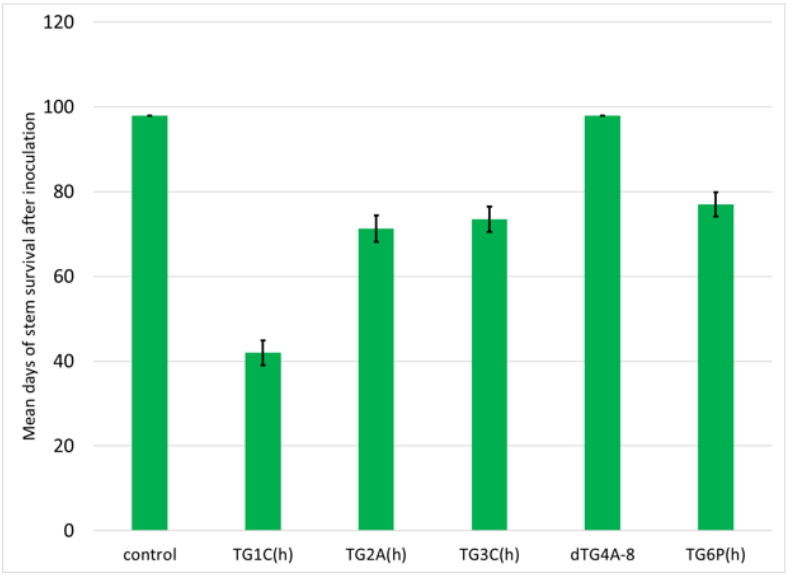


Figure 14. Virulence as measured by days of survival post-inoculation, for the portion of the stem distal to the inoculation site.

Chapter 3: Conclusions, Reflection and Future Directions

General Conclusions

The objectives of this thesis research are laid out in Chapter 1. Had all six target genes been as easy to disrupt in *Cp* as the *TG4* gene, these objectives could have been accomplished over the past two and a half years. However, unexpected challenges, particularly, in isolating monokaryon mutants from heterokaryon cultures, slowed the progress of the project. So far, we were only completely successful in knocking out *TG4* and analyzing impact of *TG4* deletion on fungal phenotype and virulence. As of April 2019, efforts to isolate additional *Cp* knockout strains continue. In addition, ectopic expression and silencing (via HIGS) of these TG homologs from PM fungi (i.e. *Gc*) in Arabidopsis is also underway, but there are no results to report at this stage. Nevertheless, the four objectives of this ambitious project have partially been accomplished, setting the stage for more thorough research in the future.

The use of Cp as a surrogate for genetic study of conserved genes in biotrophic PM fungi

The methods we used to select genes of interest that have homologues in *Cp* and *Gc* did yield six interesting candidate genes, which, based on detailed literature research and analysis, appear to potentially play roles in pathogenesis, either by acting on the plant host, or by enabling fungal adaptation to the host. The genetic data we obtained for the one gene were successfully knocked out, *TG4,* demonstrates that the gene plays a role in *Cp*'s virulence against chestnut, as shown by reduced canker size caused by the *TG4* knockout strain in the *in planta* assays (figures 12-14).

Whether the remaining five *Cp* target genes contribute to virulence remains unknown due to the lack of corresponding monokaryon *Cp* mutants. Nor is it clear whether the *TG4* homologue in *Gc* plays a similar role in virulence. If the results from the HIGS experiment underway are positive, it will provide more evidence that the "surrogate" approach taken in this research is useful for functional characterization of conserved fungal pathogenicity genes in genetically intractable biotrophic fungi such as PM.

Identification of pathogenicity genes in plant-pathogenic fungi

Although homologues of *TG1-TG6* have been studied in yeast, they might have undergone functional diversification and play distinct and important roles in host colonization in plant pathogenic fungi. *TG1-TG5* all appear to serve as auxiliary regulatory genes that help pathogenic fungi adjust to conditions in a live host, while the exact function of *TG6* remains a mystery. If pathogenicity is broadly defined as the ability of a pathogen to overcome the defenses of its host, establish itself and obtain nutrients, and reproduce there, then the approach we have taken may help shed light on previously unstudied aspects of the host-pathogen relationship. In this sense, *TG1-TG6* warrant further study in other plant-fungal pathosystems.

Improving our understanding of the Arabidopsis-Gc *pathosystem*

As of April 2019, efforts were underway to overexpress and silence *Gc* homologues of target genes using genetically modified Arabidopsis plants. Results, when available, may reveal new information about mechanisms of pathogenicity in *Gc*.

The nature of the six target genes, as genes that help the fungus respond to host-induced stress, has improved our understanding of the types of genes involved in colonization of the host, and changes the scope of what we could define as a pathogenicity gene.

Identifying new targets for HIGS in American chestnut

This research has added six new potential targets in the *Cp* genome for silencing by means of HIGS in transgenic American chestnut. *TG4* apparently contributes to *Cp* virulence in chestnut, and the possibility that *TG5* may be essential in *Cp* suggests that it may be an excellent target for silencing as well. If results from ongoing HIGS experiments demonstrate that transgenic Arabidopsis plants expressing siRNA targeting any of the *Gc TG1* to *TG6* genes exhibit resistance to *Gc*, it may be worthwhile to direct future efforts towards engineering *Cp* resistance in American chestnut by targeting these target genes by HIGS.

Specific conclusions concerning the role of TG4 *in Cp*

The *TG4* knockout strain showed a difference in phenotype compared to the wild-type DK80 parent strain *in vitro*, as well as reduced virulence *in planta*. The research conducted within the scope of this study does not point to an exact mechanism for TG4 protein's activity, but these results, combined with the previous characterization of the TG4 homologue in *Sc*, SEC66, offer some clues.

Morphological differences between the *TG4* knockout and DK80 *in vitro*, suggest that the gene is involved in multiple cellular processes. For example, the lack of zonal growth in the *TG4* knockout indicates that deletion of *TG4* impairs *Cp*'s ability to sense or respond to light signals. Given its localization in yeast at the ER membrane, it is more likely that *TG4* is involved in the delivery of light-sensing proteins to the cell membrane or extracellular space than that it is involved in light sensing itself. The relative lack of pigment in the *TG4* knockout may also result from impaired light sensing, or simply an inability to deliver pigments or the enzymes that produce them to the right cellular locations. The disorderly growth of dTG4A-8 hyphae may also be due to sensory impairment of some kind. In addition, the rapid but sparse radial growth of *TG4* knockout mycelia compared to the wild-type is typical of how fungi respond to low-nutrient environments (Jennings and Lysek 1999, pg.13), suggesting that deletion of *TG4* impairs *Cp*'s ability to sense or take up nutrients. These disparate phenotypic changes may all be explained by the disruption of delivery of multiple specialized enzymes with a variety of functions.

The reduction in fungal virulence against chestnut tissue resulting from the deletion of the *TG4* gene may be the result of impairment in delivery of the same proteins that are responsible for the changes in phenotype, or in the delivery of other proteins. The fact that dTG4A-8 could not even match DK80 in radial growth on CBA medium as it did on synthetic media is notable because it indicates that absence of the *TG4* gene impairs the fungus's ability to overcome the effects of water-soluble, growth-

inhibiting constituents of chestnut bark. This is consistent with the idea that *TG4* is important for adaptation to the host.

Further study will be required to determine which TG4-interacting proteins are responsible for the loss of virulence. However, the results of *in vitro* and *in planta* observations of the *TG4* knockout strain suggest that *TG4* would be a good target for engineering novel forms of resistance using HIGS in transgenic American chestnut. Impairment of the gene results in a reduction in virulence but does not fundamentally impair the fungus's ability to grow as a saprophyte or to produce asexual spores. HIGS -based resistance targeting *TG4* could help artificially establish a host-pathogen equilibrium between American chestnut and *Cp*.

Further research and future directions

Closing the loop on work underway

The results of the research described in this thesis demonstrate that the method used to screen for candidate virulence-related genes in two related fungi was sound. Additional small stem assays to compare the *TG4*-knockout *Cp* strain to the parent strain, DK80, will be done in the coming summer. Identification of the role of *TG4* in virulence in *Cp* provides an opportunity to develop resistance against *Cp* in transgenic chestnut trees using HIGS. These promising results encourage continued efforts to obtain monokaryon knockout strains for the remaining target genes, followed by *in vitro* and *in planta* assays.

Determining the role of TG4 *and other target gene homologs in* Gc

We have made DNA constructs to overexpress *Gc TG4* and *GcTG6,* fused with green fluorescent protein*,* and introduced them into Arabidopsis via Agrobactgerium-mediated transformation. We will observe the transformed plants using fluorescent microscopy to pinpoint the cellular location of *Gc* TG4 and *Gc* TG6 proteins, and to observe whether the proteins have a toxic effect on the plant host.

We have also prepared another construct to test the HIGS efficacy against *Gc TG5*. A *Gc TG5* gene fragment has been cloned in the binary vector pK7WlWG2(I) which is designed for RNAi applications such as HIGS. HIGS transgenic Arabidopsis plants and isogenic wild-type plants will be inoculated with *Gc* spores to see if silencing *Gc TG5* homologue results in resistance.

Exploring differential expression of Cp *target genes*

While our selection criteria included upregulation by two-fold or greater in haustoria for the *Gc* homologues of the target genes, we do not know whether or how expression of the *Cp* target genes changes when the fungus is growing on its host. This could be accomplished by quantitative RT-PCR or RNA-seq. This information would help clarify whether the target genes perform basic housekeeping functions, or whether they are specifically involved in adapting and colonizing the host.

Characterizing subcellular localization of target proteins in Cp

Once we have successfully knocked out target genes, and know the right parameters for repeating the process, we can study the *Cp* target protein *in situ*. For example, the best means to study the subcellular localization of a target *Cp* protein is to replace them with GFP-tagged versions and observe where they accumulate under a fluorescence microscope. Using the same techniques, we can also replace the *Cp* target genes with their homologues from *Gc* and assess whether and to what degree the *Gc* genes can functionally complement the loss of their *Cp* homologues.

On to transgenic chestnut

A long-term goal of this project is to engineer *Cp* resistance via HIGS of key *Cp* pathogenicity or virulence genes in transgenic chestnut. The potential advantages of HIGS over the use of exotic transgenes in chestnut include the fact that, by targeting genetic sequences specific to the pathogen, there is a lower risk of off-target effects in the host or in the ecosystem. In addition, multiple dsRNA constructs targeting different pathogen genes could be included in a single dsRNA-encoding gene, greatly reducing the possibility that the pathogen could adapt to overcome the resistance (Ghag *et al*, 2014; Nowara *et al*, 2010; and Weiberg *et al*, 2014).

The State University of New York's College of Environmental Science and Forestry (SUNY-ESF) has developed methods to genetically transform American chestnut, but they are complex and time-consuming (Newhouse *et al*, 2014; Welch *et al*, 2007). To produce a single seedling from an embryonic chestnut cell, takes multiple simultaneous attempts over a period of 12-18 months (Bruce Levine – Linda

McGuigan personal communication, September 2018). It is desirable to find ways to identify and test transgene constructs before undertaking such laborious efforts.

The research described in this thesis has helped identify one promising gene for silencing in *Cp, TG4*. Planned work to silence the *Gc* homologues of these genes by HIGS in Arabidopsis may provide confirmation of other promising targets. The next step would be to test if *Cp* is susceptible to siRNA-mediated gene silencing, which has yet to be demonstrated by any laboratory. A technique for exposing fungi *in vitro* to siRNAs is described in Ghag *et al* (2014), as a "fungal inhibition assay." Ghag *et al* designed dsRNA constructs based on exonic regions of genes of interest in the banana pathogen *Fusarium oxysporum* f.sp. *cubensis*. The researchers had the dsRNA commercially synthesized, and then incubated *Fusarium* spores with the dsRNA in specialized buffers. Mycelia grown from these spores were then examined *in vitro*, and showed the morphological and growth defects predicted. This screening process allowed researchers to confirm that their genes of interest could be silenced by RNAi before going to the effort of developing transgenic plants. It would be advisable to do a similar screening with *Cp* prior to developing HIGS transgenic chestnut tissue or trees. *Cp* cultures silenced in this manner could be tested *in vitro* and in chestnut using the same assays used for knockout strains. If *in vitro* silencing of specific *Cp* target genes results in reduced virulence, then it would make sense to try HIGS in transgenic Chestnut trees for developing a novel form of resistance to *Cp*.

Reflections on methodology

The conclusions described in this chapter are based on results achieved over two and a half years. It was relatively straightforward to delete and replace the *TG4* gene in *Cp* and observe interesting and informative changes in phenotype and virulence. For various reasons, we have not yet isolated pure knockout strains of *Cp* for any of the other target genes.

The methods used are described in chapter 2 for the benefit of future researchers who may wish to follow up on this work or adapt it to some other purpose. We have attempted to describe one or more approaches to achieve success at each step of both the genetic transformation of *Cp* and of the assays used to observe changes in fungal phenotype and virulence. However, the reader will also see that the process was not always straightforward. For that reason, this section highlights some of the pitfalls encountered so that future researchers will be best prepared for unexpected obstacles.

DNA Amplification methods will vary by target gene

The target genes and associated DNA fragments were idiosyncratic in their behavior. It was difficult to optimize PCR methods to create gene disruption constructs, and required significant, time-consuming trial and error. The efficiency of amplification varied wildly depending on which target gene was being manipulated. There was no universal PCR protocol that was effective for overlapping PCR or for final PCR amplification of the full-length gene disruption constructs for all target genes. In fact, variation in results for different target genes seemed related to the genetic sequence

itself, as it was not possible to achieve uniform results simply by changing DNA concentration, temperature, reaction times or other parts of the PCR protocol. For those gene disruption constructs which we chose to amplify as plasmid DNA in *E. coli* bacteria (i.e. all except *TG5*), we also achieved markedly different results using different combinations of vectors and competent cells for different target genes. The amplification methods described in table 3 represent what worked for us, but further experimentation may produce better results.

Selection methods for knockout strains must be optimized for the target gene

It is unclear why the selection methods described in Churchill *et al* (1990) worked so readily for *TG4* knockouts but failed for all others. In all cases (except *TG5*, which we believe may be an essential gene in *Cp* spheroplasts), we remain optimistic that it is possible to produce monokaryon knockout mycelia, given enough time. The spheroplast preparation, transformation and regeneration methods described in chapter 2 worked well, and readily produced heterokaryon mycelia, containing both wild-type and knockout nuclei, for all but *TG5*. For *TG1*, *TG2*, *TG3* and *TG6*, however, we got bogged down in attempts to recover monokaryon mycelia from single spore or hyphal tip cultures generated from the heterokaryon mycelia. This final stage of the homologous gene replacement process, the isolation and culture of monokaryon knockout colonies, is the most challenging.

Serial dilutions are important for both spheroplasts in regeneration medium, and spore suspension in the generation of single spore colonies. Cells that produce the hph enzyme will create a hygromycin-free zone in the medium immediately around them

into which hyphae from neighboring spheroplasts or spores can grow. It is important to find a dilution that will put spheroplasts far enough apart that wild-type hyphae will not find hygromycin-free zones. We found that transferring mycelial plugs from solid medium to liquid media, in which hygromycin can diffuse better, enabled us to screen out wild-type mycelia, but we could not use it to distinguish between heterokaryon and monokaryon knockouts.

Collecting spores for the production of single spore colonies also proved challenging. It is necessary to wait at least two weeks for *Cp* grown *in vitro* to produce fruiting bodies that ooze asexual spores. Once they do, one can use a pipette to deliver a water droplet to the tips of oozing fruiting bodies and then transfer the droplet to a larger volume of water for dilution. However, we recovered heterokaryon DNA from numerous *TG2* and *TG6* knockout colonies that had grown from such diluted spore suspension. As *Cp* asexual spores carry only one nucleus, this should not have happened – unless either dilution was not sufficient, and wild-type spores were germinating in the hygromycin-free zones surrounding mutant spores and then fusing with them by anastamosis, or we were picking up small multinucleate hyphal fragments along with the spores, and these were growing into heterokaryon colonies. In either case, we would have expected to recover at least some monokaryon colonies, but we did not, leaving an open possibility that all of the target genes aside from *TG4* are essential in *Cp*.

It is also possible that if a given target gene is not constitutively expressed, a knockout strain with the hph gene stably integrated into its genome may still not produce enough hph enzyme to grow in hyg+ medium unless the medium also contains the factors necessary to induce gene expression. While the hph marker cassette contains its own constitutive promoter, the location of the target gene may also determine whether or not the whole cassette is actively transcribed. Our limited efforts to experiment with induction media were inconclusive—the medium itself clearly inhibited hygromycin, and it was not clear whether the medium also induced spore germination better than EPC medium. In any case, it may not always be feasible even to guess whether a particular target gene is constitutively expressed or needs to be induced, and if so, what induces it.

Hygromycin may not be the ideal selective agent

Other antibiotics or other selection methods may be less problematic than hygromycin. We learned during the course of this research, that *Cp* naturally has some degree of tolerance for hygromycin. We discovered this when we recovered *TG5* wild-type mycelia from media containing 30 mg/mL of hygromycin. Raising the hygromycin dosage to 50 mg/mL reduced the number of wild-type colonies but did not eliminate them. The *TG4* knockout, which grew perfectly well on EPC medium with 100 mg/mL of hygromycin when started from mycelial plugs, showed very delayed germination when inoculated in the form of spores. No other Cp strain would grow on EPC+hyg100 at all. This suggests that spores are more sensitive to hygromycin than hyphae. Our only successful knockout used a dosage of 50 mg/mL,

but this may not be the right dosage for all potential targets. As noted above, the selective dosage may depend on the expression level of the target gene.

Cp tolerance for hygromycin also varies by strain. Figure 15 shows several strains of *Cp* growing on medium with 50 mg/mL of hygromycin. The weakly virulent standard *Cp* strain SG2,3 shows greater tolerance for hygromycin than DK80 or DK80's parent strain EP155.

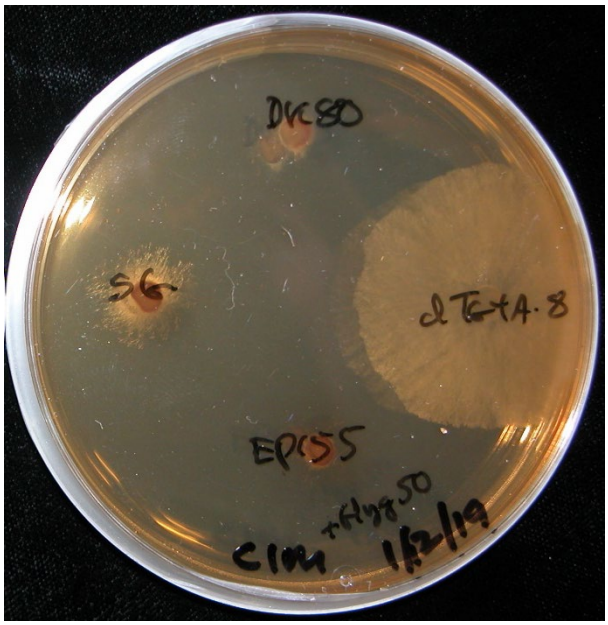


Figure 15. Four isolates of *Cp* growing on Chestnut Induction Medium with 50 mg/ml of hygromycin. The dTG4A-8 isolate carries an introduced hygromycin resistance gene, while the others do not. Wild type SG2,3 nevertheless shows an ability to survive and grow in the presence of hygromycin.

Contamination can mimic hygromycin resistance

Even following standard laboratory procedures and working under a laminar flow hood, hygromycin-resistant airborne bacterial and fungal contaminants frequently appeared in our cultures. We were able to identify them by culturing them, extracting

genomic DNA, amplifying the ITS and RPB2 regions and searching the sequence ID by BlastN. We identified *Aspergillis nigra*, *Penicillium restrictum*, *Sporothrix* and *Lasiodiplodia* species, and were unable to identify several other fungal or any bacterial contaminants. Some contaminants, including the bacteria and the *Penicillium* fungi, were not just insensitive to hygromycin, but capable of neutralizing hygromycin in the media around them and allowing non-transformed *Cp* colonies to grow. We spent considerable time attempting to separate *Cp* colonies from contaminants, culturing them, extracting DNA and performing genomic analysis via PCR only to learn that they were wild-type DK80 colonies.

It was also quite difficult to separate some contaminants from *Cp*. Several *Penicillium* isolates grew parasitically on *Cp* hyphae, rather than on the medium (figure 16), though they could also be cultured on nutrient-rich medium. These fungi were not always visible until we attempted to grow single spore colonies, at which time we would discover that most of the spores which germinated were contaminants.

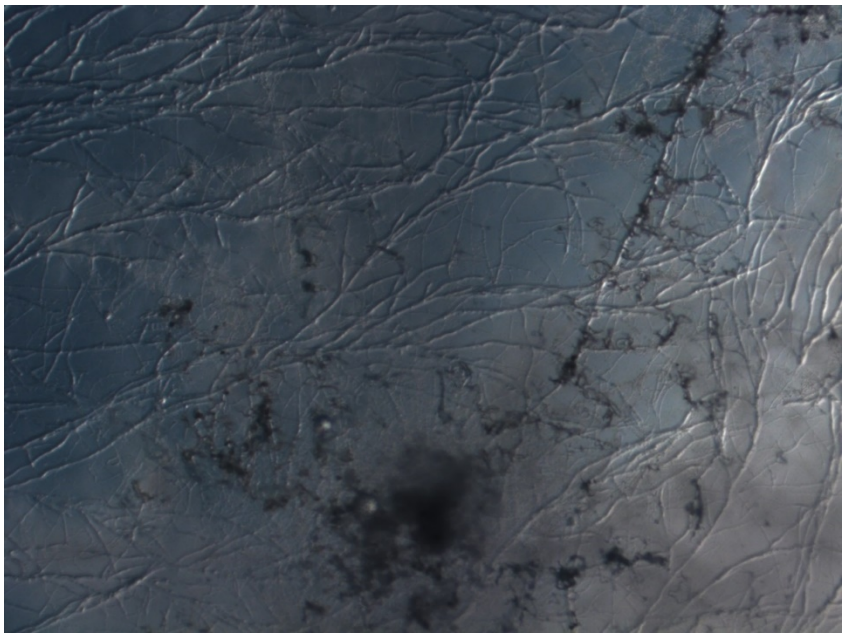


Figure 16. Dark hyphae of *Penicillium* c.f. *restrictum* fungi growing parasitically on the larger clear hyphae of *Cp*.

To manage the risk of contamination, we recommend growing isolates of all important strains developed during the course of research on filter paper, and storing the filter papers dry in a -20°C freezer, in case it is necessary to go back and make new inoculum from an uncontaminated source. Standard cultures stored at 4°C are not reliable after more than 2-3 months, as they continue to grow, lose virulence and can get contaminated in storage. For heterokaryon strains, we observed that the ratio of mutant to wild-type nuclei tended to decline over time in storage, as reflected in the relative brightness of bands appearing in gel electrophoresis.

All inoculum should be the same age

The morphology and virulence of *Cp* mycelia will change over time, and with successive subcultures. During the course of this research, we learned that DK80, and presumably any daughter strains developed from it, ages and loses virulence

unusually fast. Whether this is due to the impaired non-homologous end joining pathway that makes DK80 so efficient for transformation, or to some other factor is unknown. DK80 begins to exhibit abnormal phenotype (wavy margins, intense pigmentation and slow growth – figure 17) only 2 – 3 subcultures after being started from spores. Our use of an older DK80 subculture resulted in a near complete failure to produce cankers in a small stem assay attempted in summer 2018. For all *in vitro* and *in planta* observations, *Cp* isolates should be started from spores on PDA or EPC medium, and subcultured 1-2 times before use in inoculation assays.

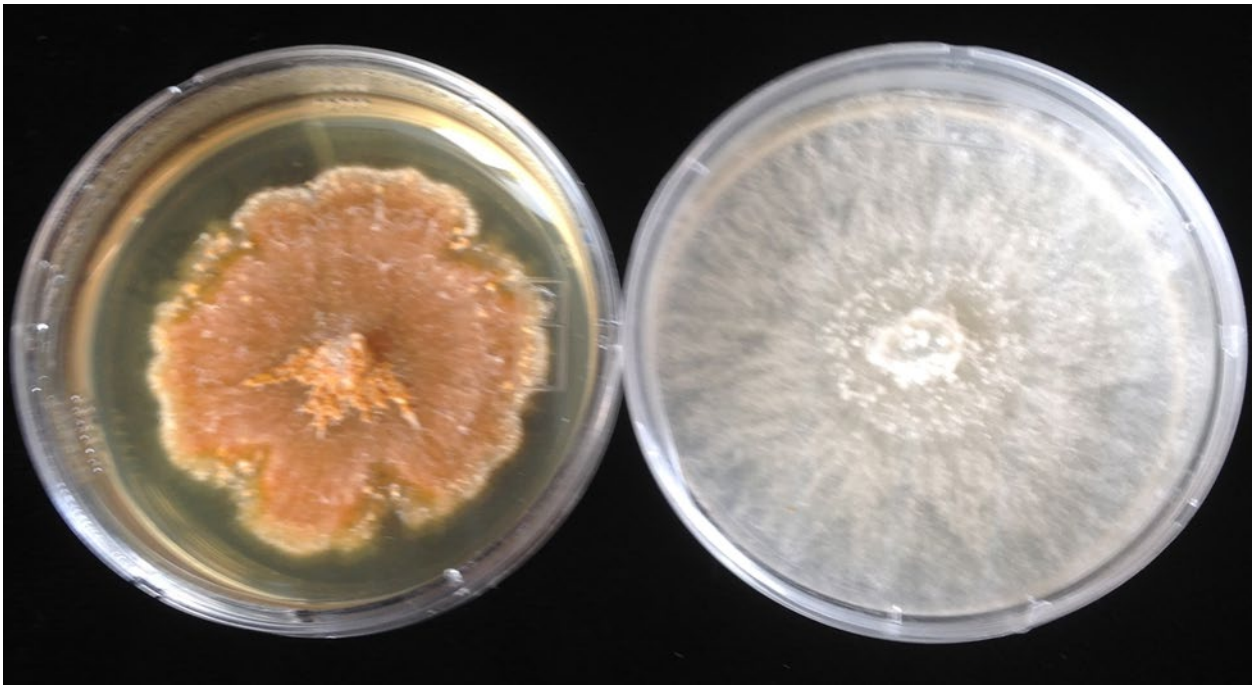


Figure 17. Two DK80 subcultures from the same parent culture, growing on PDA medium, inoculated on the same day. The one on left was started with a mycelial plug, while the one on the right was started from spores collected from the parent colony.

Appendix I

Fungal Growth Media Used in this Study
(all recipes given for 1 liter volume)

Water Agar
20 g Difco Bacto Agar
1 liter dH20

Autoclave at 120°C for 20 mins.

Potato Dextrose Broth/Agar (PDB/PDA)
24 g Difco Potato Dextrose Broth
15 g Difco Bacto Agar (omit for broth)
1 liter dH20

Autoclave at 120°C for 20 mins.
(To make acidified PDA, add 4.5 ml 25% lactic acid after medium cools to 50°C)

Endothia trace elements solution:
60 mg H3B03
140 mg MnCl2 x 4H20
400 mg ZnCl2
40 mg Na2MoO4 x 2H20
100 mg FeCl2 x 6H20
400 mg CuSO4 x 5H20

Autoclave at 120°C for 20 mins
(FeCl2 will precipitate from solution. Stir before using.)

Endothia salt solution:
24 g NH4N03
16 g KH2PO4
4 g Na2SO4
8 g KCl
2 g MgSO4 x 7H20
1g CaCl2
Add dH20 to volume of 1 liter

Autoclave at 120°C for 20 mins

***Endothia parasitica* minimal (EPM) broth/agar**:
54.5 ml Endothia Salt Solution
8 ml Endothia Trace Elements Solution
10 g Glucose
15 g Difco Bacto Agar (omit for broth)

Autoclave at 120°C for 20 mins
2 mg Thiamine hydrocholoride (added as a filter-sterilize aqueous solution after autoclaving)

***Endothia parasitica* complete (EPC) broth/agar**:
54.5 ml Endothia Salt Solution
8 ml Endothia Trace Elements Solution
10 g Glucose
25 g Difco yeast extract
75 g Difco malt extract
15 g Difco Bacto Agar (omit for broth)

Autoclave at 120°C for 20 mins
2 mg Thiamine hydrochloride (added as a filter-sterilize aqueous solution after autoclaving)

Chestnut bark extract (CBE):
100 g dried bark strips, approximately 5 mm wide and 50mm long, stripped from washed, surface disinfested chestnut stems, 3-10 cm in diameter. (Note: fungus grows better on winter harvested bark, and Cp grows better on bark from American chestnut than Chinese chestnut)
1 liter dH20
Allow bark strips to steep at room temperature for 24 hours, then allow another 24-48 hours at 4°C to prevent fermentation.
Filter through paper filter, then autoclave at 120°C for 20 mins
(can be pH adjusted with NaOH, HCl or lactic acid)

Chestnut Bark Agar (CBA):
Prepare
500 ml dH20
20 g Difco Bacto Agar

And separately prepare

Chestnut Bark Extract

Autoclave in separate flasks at 120°C for 20 mins. Allow media to cool under hood to 50°C, then mix thoroughly by swirling and pour. (Note: the tannic acid in CBE will hydrolyze the agar if autoclaved together, resulting in a soft, semi-solid medium.)

Cp Induction Medium:

Prepare

500 ml dH20
54.5 ml Endothia Salt Solution
8 ml Endothia Trace Elements Solution
5 g Sucrose (do not use glucose, which can inhibit induction of some genes)
3.9 g Difco Malt Extract
20 g Difco Bacto Agar

and separately prepare

Chestnut Bark Extract

Autoclave in separate flasks at 120°C for 20 mins. Allow media to cool under hood to 50°C, then mix thoroughly by swirling. Then add:

2 mg Thiamine hydrochloride (added as a filter-sterilize aqueous solution after autoclaving)

Pour before it solidifies. (Note: thiamine is temperature sensitive and will break down if media is reheated in the microwave. Also, the tannic acid in CBE will hydrolyze the agar if mixed and then brought to a high temperature, resulting in a soft, semi-solid medium.)

Appendix II
Spheroplast Preparation, Transformation and Regeneration

A. Buffers and media needed

Osmotic Medium (for 500 ml):

1.2M $MgSO_4$ (use $MgSO_4 \bullet 7H_2O$ [148g /500ml] which dissolves easily in water, combining anhydrous $MgSO_4$ with water is very exothermic) in 10mM NaH_2PO_4 (use $NaH_2PO_4 \bullet H_2O$, 0.69g /500ml) ; adjust to pH 5.8 with 1M Na_2HPO_4 (0.5M Na_2HPO_4 is easier to prepare, add the chemical slowly to water). Note that a precipitate forms while adjusting the pH so add the 0.5M Na_2HPO_4 slowly; 50-100ml may be necessary to reach pH 5.8. Filter sterilize and store at 4°C.

Digestion Buffer (for 50 ml) (Note: Prepare fresh for each use):

50 ml osmotic medium
500 mg bovine serum albumen
100 mg lysing enzyme from Trichoderma harziana
1,000 mg vintastepro enzyme (beta D glucanase)
500 ul of beta-glucuronidase

Add ingredients in order listed, and allow BSA to dissolve thoroughly before adding the next ingredients.

Trapping Buffer

0.4 M sorbitol (36.4 g/ 500ml, or 200ml of 1M sorbitol/500ml) in 100mM Tris-HCl, pH 7.0 (50ml of a 1M solution); autoclave and store at 4°C.

STC

1M sorbitol (91.1g/500ml) in 100mM Tris-HCl, pH 8.0 (50ml of a 1M solution) and 100mM $CaCl_2$- dihydrate (7.35g/500ml); autoclave and store at 4°C.

PTC

40% polyethylene glycol 4000MW (80g / 200ml), 100mM Tris-Hcl, pH8.0 (20ml of a 1M solution), 100mM $CaCl_2$-dihydrate (2.94g /200ml); autoclave and store at room temperature.

Spheroplast Storage Buffer

4 parts STC, 1 part PTC, 0.05 parts DMSO

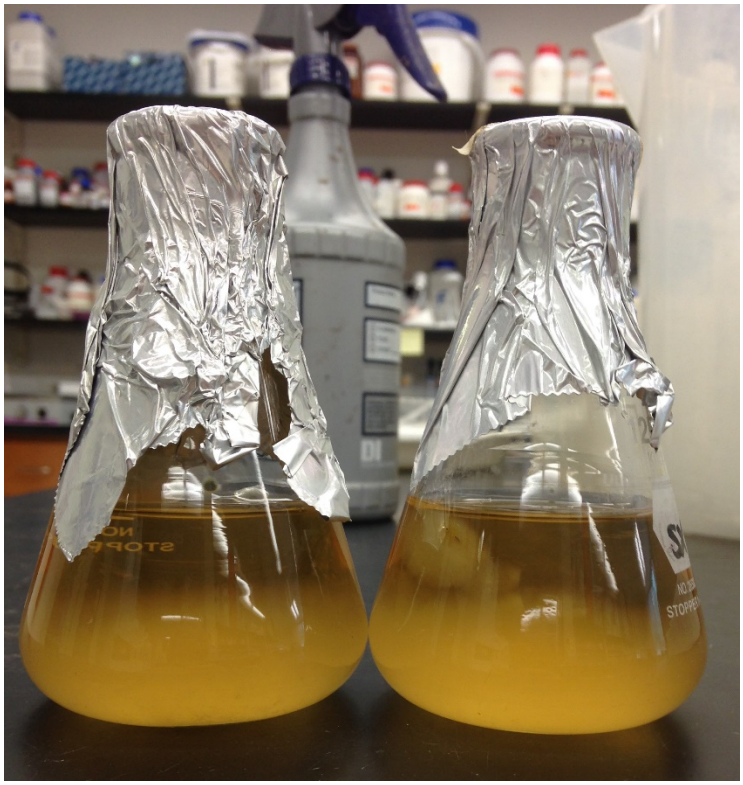
Regeneration Medium (400 ml)

390 ml dH_20
171 g sucrose
0.5 g yeast extract
0.5 g casein hydrolysate
8.9 g Difco Bacto agar

Dissolve the sucrose in the dH_20, add the other ingredients, with agar added last. Autoclave at 120°C for 20 minutes..

B. ***Cryphonectria parasitica*** **spheroplast preparation:**

1) Use a pipette tip to "cotton ball" **Cp hyphae** from colonies grown on **PDA medium**. Transfer-to 1.5 ml centrifuge tubes. Wash with dH20 and grind with plastic micro-pestle (about 50 twists of the pestle will create a suspension



of finely fragmented hyphal tissue). Inoculate 100 ml PDB medium (1 good cotton ball from one fresh PDA culture should be enough). Incubate the culture on bench top over 3 days. You should have white clouds of mycelia. If it coats the bottom of the flask, or turns orange, try again starting with the youngest hyphae you can get.

2) When ready for spheroplast preparation, make fresh **Digestion Buffer**

When preparing, add **osmotic medium** to tube, add dry ingredients starting with BSA, and allow it to dissolve before adding the other dry ingredients. Add beta-glucuronidase last. Mix by inversion.

3) Place mycelium suspension in sterile, 50 ml disposable centrifuge tubes and spin down at room temperature at 4000 rpm for 5 mins. Pour off supernatant. Combine contents so that mycelia from 100 ml of media are in one 50 ml tube. Wash by gently adding ~25 ml of **autoclaved dH20.** Spin down at 4000 rpm for 3 min. Repeat wash and spin. If necessary to remove moisture, you can pour the mycelia onto autoclaved filter paper and blot.

4) Re-suspend pellet in 25-30 ml of **Digestion Buffer**, screw the lid on tight, and incubate horizontally at 30C overnight at a very low speed, e.g. 50 rpm. At the end of about 16 hours, you should have homogenous cloudy suspension. If you have a lot of lumps or the mycelium appears unchanged, it has not digested properly.

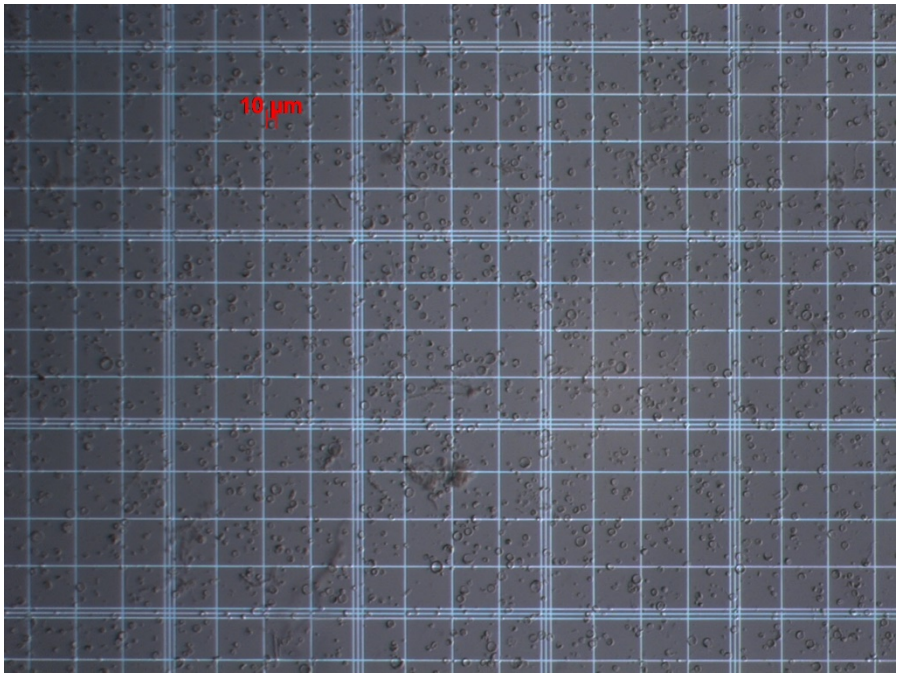
5) Very carefully add cold, sterile **Trapping Buffer** to overlay the spheroplast suspension (about 25 ml, or whatever amount is required to fill the tube). Be careful not to disrupt the spheroplast layer, which should look like a cloudy area of trapping buffer that is densest closer to the margin with the digestion buffer..) Use a 25 ml pipette to gently add the trapping buffer to the side of the tilted 50 ml tube. Spin down again at room temperature at about ~4000 rpm)

6) Collect the spheroplasts (the cloudy layer) at the interface. Transfer to a new 50 ml tube, and dilute with 2 vols of **1M sorbitol.** Gently mix thoroughly. Pellet the spheroplasts in rotor at 6,000 rpm, 4°C, 5 min.

7) Remove supernatant by aspiration. Suspend the spheroplast pellet in 100-200 ul of **STC.** If you have multiple tubes, you can use the same suspension over, transferring from tube to tube until everything is suspended and combined in one tube. You can clean the last spheroplasts from the other tubes with additional fresh STC and transfer that to your main tube. Pellet everything down again, as in step 4.

8) Remove supernatant by aspiration. Suspend spheroplasts in **STC** on ice. Use enough STC (normally 200-1000 ul) so that you have a slightly cloudy homogeneous solution. If very cloudy, dilute more.

9) Observe up to 10 ul of suspension under dissecting scope with a hemacytometer. Spheroplasts are spherical and about 5 um across, and will look under the scope like craters on the moon. See picture below. If you have very high concentrations, you can dilute until you have a final concentration of 2 x 10^8 cells/ml.



10) Dilute your spheroplast suspension with the following solution: 4 parts STC, 1 part PTC and 0.05 parts DMSO. For example: 4ml STC, 1ml PTC and 50µl DMSO. Freeze in 50 ul aliquots in cryovials and store at-80°C.

C. <u>Spheroplast transformation and regeneration</u>

1) If spheroplasts are frozen, quickly thaw in a 37º C water bath until a 2mm diameter ice crystal remains, then place on ice. Spheroplasts should be at a concentration of ~ 2 x 10^7 cells/ml.

2) Pre-cool 1.5 ml tubes on ice, one for each transformation, plus a control. Add 5-10µg of DNA in a volume of 10µl to each tube and 10µl of TE buffer to one other for a control.

3) Add 50µl of spheroplasts to each tube, mix gently, and chill on ice for 30 min.

4) Add 500 ul of **PTC** to each tube, mix gently and incubate at room temperature for 25 min. THIS IS THE TRANFORMATION STEP.

5) Add 1ml of **STC** to each tube, mix gently.

6) Evenly distribute the reaction mixtures by droplets onto empty, labeled petri dishes. Make serial dilutions to ensure the separation of individual transformants. A suggested series is 2µl, 20µl and 200µl. Pipet 11 ml of **Regeneration Medium** at 48º C onto each plate and swirl to mix.

Incubate on bench top for 16-18 hours, then add 11 ml of **Regeneration Medium containing 100µg/ml Hygromycin B (or other antibiotic)** at 48º C as a top layer. Do not add the top layer to the cells mixed with the TE buffer unless you wish to test the antibiotic. (The previous two steps can also be combined with one stem involving 22 mlRegeneration Medium+antibiotic.) Note that the regeneration medium is sticky and difficult to handle. Keep it at 48-50 C in a water bath near where you are doing the inoculations, to prevent it from congealing. Especially if you are doing the two-layer method, gaps or bubbles can allow non-transformed fungus to grow around the selective medium layer.

7) Incubate on bench top for 3-5 days until hyphae can be seen growing through the top layer. Pick individual putative transformants to PDA plates containing antibiotic. Also transfer hyphae from TE control plates to PDA without antibiotic to compare the phenotype to that of the putative transformants.

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