AN ABSTRACT OF THE DISSERTATION OF

Michael Ian Gordon for the degree of Doctor of Philosophy in Molecular and Cellular Biology presented on December 13, 2023.

Title: Host-Induced Gene Silencing in Black Cottonwood for Control of Septoria Canker: Efficacy and Non-Target Impacts.

Abstract approved: __________________________________________________________

Steven H. Strauss Jared M. LeBoldus

Septoria canker remains the most important disease of poplars in intensively managed forest plantations. Genetic resistance has long been considered the best way to manage for this disease. Transgenic resistance mediated by RNA silencing against pathogens and pests (HIGS: host-induced gene silencing) has shown promise in other pathosystems but has never been tested in a forest tree against a fungal pathogen. The effects of HIGS are expected to be specific to the target organism and closely related taxa; however, this expectation has never been evaluated empirically against fungal pathogens under field conditions. In addition, the effects of plant transformation and associated in vitro propagation and sterile culture on subsequent microbial community assembly have never been previously evaluated, but may be an important unintended effect of HIGS technology. To answer these questions, we studied the direct effects of synthetic double-stranded RNAs (dsRNAs) on the target pathogen in axenic culture, we created transgenic poplars that contained HIGS transgenes, and we evaluated their disease resistance in the greenhouse, and their foliar fungal community composition in the laboratory and the field.

Our first study explored HIGS as a novel means to engineer resistance to Septoria canker caused by the fungal pathogen Sphaerulina musiva. HIGS transgenic poplars expressing double-stranded RNA (dsRNA) that targeted either or both S. musiva CYP51 (P450
lanosterol C14α-demethylase) and DCL (dicer-like) genes were screened for resistance to stem canker disease in two greenhouse inoculation trials. We did not detect statistically significant differences in disease severity between transgenic lines and wild-type controls. The correlation between variation in greenhouse-expressed disease severity and transgene expression was statistically significant for HIGS events targeting DCL but not for HIGS events targeting CYP51.

*In vitro* studies with synthetic dsRNAs tested: (1) *S. musiva*’s capacity for uptake of environmental dsRNA; (2) effects of *in vitro* silencing of CYP51 and DCL on fungal growth and target transcript abundance; and 3) persistence of dsRNA in culture. We were unable to detect uptake of fluorescently tagged dsRNA *in vitro* with confocal live imaging, though using the same methods we were able to detect uptake in another fungal pathogen known to be amenable to HIGS, suggesting a very limited capacity for dsRNA uptake by *S. musiva*. In dsRNA-treated cultures, we were unable to detect fungal growth inhibition, and RNA was rapidly degraded in the culture medium. Of five target transcripts tested after dsRNA treatment, only one had significantly impaired expression. These results, together with our disease screening, suggest that HIGS and the related technique SIGS (spray-induced gene silencing with dsRNAs) are unlikely to be effective control measures for this disease.

In our second study we used ITS metabarcoding to evaluate the effects of HIGS transgenes, and the effects of plant transformation—including micropropagation, antibiotic selection, and organogenesis—on the foliar fungal microbiome of field-grown trees over two seasons. Micropropagation-derived trees grown in a greenhouse were largely uncolonized after 50 days. Once established in the field, after a single growing season, communities contained 372 operational taxonomic units (OTUs) and samples were as rich as those of trees of the same genotype that had been growing at a nearby location for several years. We did not detect any effects from micropropagation, or from the use of antibiotic selection and organogenesis during *Agrobacterium* transformation, on subsequent leaf fungal communities. The expression of HIGS constructs directed against the Septoria canker fungus also had no detectable effect on any non-target fungal
taxa, including the very closely related species *Sphaerulina populicola*. Our results suggest that micropropagation, genetic transformation and associated antibiotic selection and organogenesis, and the expression of antifungal HIGS genes, had minimal, if any, impacts on subsequent foliar microbiomes of field-grown trees.
Doctor of Philosophy dissertation of Michael Ian Gordon presented on December 13, 2023

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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Michael Ian Gordon, Author
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CONTRIBUTION OF AUTHORS

Steven Strauss, Jared LeBoldus, and Posy Busby conceived the ideas behind chapters two and three, guided analysis, and edited both manuscripts.

Cathleen Ma oversaw plant transformations, culture maintenance, and propagation for transgenic lines used in chapters two and three.
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1. Introduction

1.1 General state of forest health

Pathogens and insect pests are pivotal factors that shape the health of trees and forest ecosystems. Forest health can be defined by the extent to which forest ecosystem processes are functioning within natural historical variability (Raffa et al., 2009). Functioning forest ecosystems are important because they provide numerous ecosystem services that humans depend on (Olander et al., 2015). These include provisions of renewable materials, medicines, food, habitat for wildlife, and fresh water (Shvidenko et al., 2005). Additionally, humanity's well-being depends on healthy forests to sequester and store carbon, distribute water resources, regulate climate, and to form and retain soil (Ellison et al., 2017). Healthy forests also hold spiritual, aesthetic, and recreational value (Cooper et al., 2016). However, the ability of forests to deliver ecosystem services is challenged by many growing biotic and abiotic stressors (Seidl et al., 2016). While disturbances from fire, insects, and disease are recognized as essential components of forest ecosystem function, anthropocentric factors have increased the severity and frequency of disturbance and led to new potent interactions of biotic and abiotic stressors that can drive forests beyond the limits of their resilience (Millar and Stephenson, 2015).

Some of the greatest threats to forest health are from non-native insects and pathogens (Kirst et al., 2014; Lovett et al., 2016). The spread of exotic species has been facilitated by increased global trade and human mobility (Early et al., 2016). Invasive pathogens and insects are typically devastating because their newfound hosts lack resistance (Herms and Mccullough, 2014). In North America, several high-impact species have been introduced. Chestnut Blight caused by the fungus Cryphonectria parasitica was introduced through imported Asian varieties of chestnut and functionally eliminated the keystone species American chestnut from eastern forests (Anagnostakis, 1987). Also introduced from imported plants, white pine blister rust caused by Cronartium ribicola has caused extensive mortality in susceptible five-needle pine species, especially in forests of Western North America (Kinloch, 2003). In coastal California and Oregon, Sudden oak death caused by the introduced oomycete Phytophthora ramorum has led to
high mortality of native tanoaks (Cobb et al., 2020). Emerald ash borer was introduced to the midwestern United States through infested wood shipping containers and has since spread to both coasts where most native ash species lack resistance and succumb to infestation (Aukemea et al., 2010; Herms and Mccullough, 2014).

Threats to forest health have already been exacerbated by a changing climate (Sturrock, 2012). Abiotic stress caused by rising temperatures and increasing frequency of droughts has led to increased tree mortality due to beetle infestations (Breshears et al., 2005; Berg et al., 2006). Warmer winter temperatures and fewer cold snaps allow expansion of pests and pathogens into higher elevations and latitudes (Carroll et al., 2004; Battisti et al., 2005; Esper et al., 2007; Dukes et al., 2009). Furthermore, increases in pathogen and pest reproduction rate are also tied to a warming climate, all of which contributes to more frequent outbreaks and severity of disease (Ayres and Lombardo, 2000; Bale et al., 2002; Seidl et al., 2016). The ability of forests to withstand stress compounded by a changing climate has limits, beyond which the decline or collapse of keystone species and ecosystem services can occur (Millar and Stephenson, 2015).

One solution to ease pressures on wildland forests while meeting an increasing demand for forest products is plantation forestry (Walter 2004; Pirard et al., 2016). Although plantations can provide an alternative and sustainable source of forest products, these forests also have forest health problems (Fenning and Gershenzon 2002; Bauhus et al., 2010). Short-rotation forest plantations are predominantly a mosaic of monocultures of single clones, making them especially vulnerable to outbreaks of pests and disease (Liu et al, 2018). Despite this threat, monocultures are preferred because of simplified management (Nichols et al., 2006). Furthermore, 44 percent of global plantation area is planted with exotic species (FAO, 2020). While plantations of exotic species are isolated from their natural antagonists, yields can be affected by both native and introduced pathogens (Wingfield, 1999). The continued success of forest plantations to reduce pressure on natural forest ecosystems will depend on effective disease management measures and the ability to rapidly identify and deploy mosaics of resistant clones. It is
also likely to increasingly rely on the use of alternative species and management regimes as climate change forces them beyond their limits of adaptability.

1.2 Septoria leaf spot and stem canker disease of poplar

1.2.1 Signs, symptoms, and disease cycle

*Sphaerulina musiva* (synonym *Septoria musiva*) is an ascomycete fungus of class *Dothideomycetes* and is the causal agent of Septoria leaf spot and stem canker of *Populus spp.* and their hybrids (Bier, 1939). The disease cycle begins in spring when primary inoculum (ascospores) is released by wind and rain from pseudothecia growing on overwintered leaves and older cankers (Bier, 1939). Spots appear on infected leaves three to four weeks after budbreak (Thompson, 1941). Cankers initiate on the current season's growth and become visible as dark and sunken necrotic lesions (LeBoldus *et al.*, 2016). As symptoms progress, mature pycnidia appear as black dots in the center of leaf spots and cankers that ooze pink tendrils containing asexual spores (conidia) (Waterman, 1941). A secondary infection cycle is initiated as conidia spread by rain splash to reinfect leaves and stems (Waterman, 1941). The cycle concludes in fall with the formation of a diploid thallus on fallen leaves that will produce more pseudothecia and primary inoculum in the following spring (Bier, 1939; Thompson, 1941).

1.2.2 Infection biology

The lifestyle of *S. musiva* is classified as hemibiotrophic (Ohm *et al.*, 2012). Colonization of host tissue occurs through natural openings including stomata, lenticels, and petiole junctions (Weiland and Stanosz, 2007; Qin and LeBoldus, 2014). During the initial biotrophic phase of infection, *S. musiva* is not known to form appressoria or haustorium—structures used by other fungi (including some hemibiotrophs) to aid invasion and nutrient uptake (Horbach *et al.*, 2011; Qin and LeBoldus, 2014). Colonization is restricted to the apoplast (Abraham *et al.*, 2019) where necrotrophy eventually ensues with secretion of pectic, cellulolytic, and proteolytic enzymes that facilitate host cell death and feeding on dead and dying cells (Dhillon *et al.*, 2015).
1.2.3 Distribution

*S. musiva* is indigenous to North America with a historical distribution that is sympatric with its primary co-evolved host eastern cottonwood (*P. deltoides*) (Bier 1939; Newcombe et al., 2001). In eastern cottonwood, *S. musiva* is only a leaf parasite but is known to cause both leaf spots and stem cankers on black cottonwood (*P. trichocarpa*), balsam poplar (*P. balsamifera*), as well as many other native and exotic *Populus* species and hybrids (Bier, 1939; Ostry and McNabb, 1986; Feau et al., 2010; LeBoldus et al., 2016). Septoria canker is now found throughout much of the United States and Canada. Stem canker disease is the most serious disease of poplar plantations where susceptible clones planted in uniform stands are especially vulnerable to outbreaks (Waterman, 1954; Ostry and McNabb, 1983).

1.2.4 Threat to black cottonwood

The extensive damage to poplar plantations caused by Septoria canker (Stroble and Fraser, 1989; Ostry and McNabb, 1985; Ostry et al., 1989; Lo et al., 1995; Weiland et al., 2003; LeBoldus et al., 2009) and the invasion of *S. musiva* into the Pacific Northwest has led to concern over the well-being of wild stands of highly susceptible black cottonwood (Feau et al., 2010; LeBoldus et al., 2013; Herath, 2016). Spread of the disease into the Northwest also threatens the continued commercial use of desirable *P. trichocarpa* hybrids in regional plantations (Newcombe and Ostry, 2001). Callan et al. (2007) made the first report of *S. musiva* in the Fraser Valley of British Columbia. It has since been found in neighboring plantations and wild forests with a pattern that suggests anthropogenic spread (Herath et al., 2016). Recently *S. musiva* was found in a plantation in Boardman, Oregon (Søndreli et al., 2020). Outside of plantations, black cottonwoods occur in dense stands with high levels of spring precipitation that could facilitate outbreaks (DiFazio et al., 2011) and mortality in this dominant riparian species (Franklin and Dyrness, 1973).

1.2.5 Known resistance

Histologic and genomic studies have provided the best clues to understanding the drivers of resistance and susceptibility in *Populus*. Resistance to stem canker has been associated with a stronger and more rapid defense response that includes the formation of a thick
necrophylactic periderm at the site of infection (Weiland and Stanosz, 2007; Qin and LeBoldus, 2014). This response was delayed or absent in susceptible genotypes, suggesting *S. musiva* interference with the host immune response. Another study using expression analysis during infection linked resistance to upregulation of genes involved in oxidation–reduction, protein fate, secondary metabolism, and accumulation of defense-related gene products (Liang *et al.*, 2014). In susceptible genotypes, transcripts involved in effector recognition were upregulated which suggested an overactive hypersensitive response, that may help deter biotrophs but conversely might aid susceptibility to necrotrophic fungi. Host cell receptors were further implicated in resistance and susceptibility by a genome-wide association study of gene expression during infection in approximately 1000 *P. trichocarpa* genotypes (Muchero *et al.*, 2018). A functional allele for G-type lectin receptor-like protein kinase combined with non-functional alleles for putative immunity receptors (two receptor-like proteins and one L-type lectin receptor-like kinase) were linked to the widespread susceptibility of *P. trichocarpa* to *S. musiva*.

1.2.6 Management

Control measures to limit disease include chemical, biocontrol, cultural, and genetic based resistance. Neither chemical nor biological control measures have proven to be cost-effective at a plantation scale (Ostry and McNabb, 1983; Ostry, 1987, Gyenis *et al.*, 2003). Cultural control by removal of leaf litter harboring primary inoculum is recommended for nurseries (Mottet *et al.*, 2007), though one study found this ineffective at reducing disease the following year as inoculum can presumably be blown in from neighboring plantations and wild trees (Ostry, 1987). Planting of resistant genotypes remains the preferred strategy for disease control in plantations (Ostry 1987; Newcombe and Ostry 2001). The identification of trees resistant to invasive disease has historically relied on traditional breeding programs that have had success but can take decades (Sneizko 2006). Genome-informed breeding and novel transgenic biotechnologies such as RNA interference have the potential to increase the efficiency of future breeding efforts by enabling otherwise highly productive clones to be specifically modified for disease resistance.
1.3 Biotech in forestry and social obstacles

Biotechnology can refer to several techniques used in forestry that include traditional breeding, genome informed breeding, grafting, biological control, tissue culture, and genetic engineering (GE) (Jacobs et al., 2023). Out of all these tools, GE has faced the most extreme social obstacles to applied use in forests. Since GE of forest trees began (Fillatti et al., 1987), many traits have been developed including pest and pathogen resistance, altered lignin, herbicide tolerance, sterility, and growth traits among others (Harfouche et al., 2011). Despite the diversity of GE applications in trees, there are very few examples of GE forest trees that have been approved for release and are being grown today. Insect resistant Bt poplar is reportedly grown in limited areas of China (James, 2015), growth modified and herbicide tolerant Eucalyptus has been deregulated in Brazil (Avisar et al., 2023), and a poplar modified for increased carbon sequestration was recently planted in Georgia (Popkin and Melton, 2023). Aside from these few examples, most GE forest trees do not make it past greenhouse and field demonstrations.

Deregulation and adoption of even the most promising biotech trees is highly dependent on social factors (Chang et al., 2018).

The adoption of GE trees in forests ultimately requires a public acceptance that encompasses many groups including citizens, government agencies, companies, consumers of forest products, and environmental non-governmental organizations (Davidson et al., 1997; Sedjo, 2010). Diverse interests and viewpoints within these groups have led to lengthy or prohibitive regulation, litigation, ballot initiatives, protests, and vandalism of field trials (Floyd and Johnson, 2012; Kolodinsky and Lusk, 2018; Strauss et al., 2016).

Social science has shown that the societal response to GE forests is influenced by many factors that include environmental beliefs and ethics, perceived risks and benefits, actual and perceived subject knowledge, trust in knowledge sources, and perceptions of naturalness (Sedjo, 2006; Gutpa et al., 2012; Strauss et al., 2017; Fernbach et al., 2019, Emery, 2020). For example, a common concern voiced by the general public is the perception of GE forms of biotechnology as inherently "unnatural," often associated with
the notion of "tampering with nature" (Sjöberg, 2004; Hajjar and Kozak, 2015; Jepson and Arakelyan, 2017; Lull and Scheufele, 2017). This apprehension is seen as diminishing the wildness or natural character of forests, thereby reducing their intrinsic value. Conversely, when GE is suggested as a solution for addressing forest health, such as in the restoration efforts for the American chestnut wiped out by an invasive blight, attitudes towards biotechnology in forests tend to be more favorable (Petit et al., 2021).

One major reason for the lack of GE forest trees today is due to policies of forest certification groups that exist to promote the trade of products from sustainably managed forests (Strauss et al., 2015; FAO, 2020). About 10% of global forested area, and 28% of North American forests, are represented by two main certifiers, the Forest Stewardship Council (FSC) and the Programme for the Endorsement of Forest Certification (PEFC) (FAO, 2020). These two groups have traditionally banned plantings of GE trees, and until 2011 made no exemptions for research, which has been frustrating to scientists and forestry companies who are able to inform actual benefits and risks of the technology with extensive empirical studies (Strauss et al., 2015; Strauss et al., 2019; Conrow, 2021). Without a guarantee that GE trees could ever be grown in certified forests, developers have little incentive to invest (Strauss, et al., 2009). Recently, the FSC briefly considered direct engagement with technology developers to study the benefits, harms, and management of GE field trials to help them to codify safeguards for GE use, but has since discontinued its interest, apparently due to the highly controversial nature of GE within FSC (FSC, 2023). The reluctance by the FSC to allow GE trees in certified forests likely reflects political pressures and ideologies among some of its members that are fundamentally opposed to those of scientists who share consensus over the potential benefits of GE trees (National Academies, 2019).

### 1.4 RNA interference and HIGS

#### 1.4.1 History and Discovery

RNA interference (RNAi) was first observed in plants when Napoli et al. (1990) attempted to make a petunia more purple by overexpressing a gene involved in anthocyanin biosynthesis (chalcone synthase, (CHS)). Instead, they observed white and
variegated petunias with reduced CHS expression and hypothesized that the transgenes were "cosuppressing" the endogenous CHS gene. Similar results were observed in *Neurospora crassa* and termed "quelling" (Romano and Macino, 1992). One of the most import clues to elucidating the mechanism of RNAi was the discovery that double stranded RNA (dsRNA) was the trigger molecule for "cosuppression" and "quelling." Working with *Caenorhabditis elegans* embryos, Fire and Melo (1998) demonstrated that it was dsRNA, and not antisense or sense RNA, that was most efficient at silencing target gene expression. It took many other groups several years to work out the intricacies of the full RNAi pathway (Sen and Blau, 2006).

RNAi pathways have been found in most eukaryotes including plants, fungi, oomycetes, mammals, and insects (Shabalina and Koonin, 2008). The RNAi pathway contains three core enzymes (Hannon, 2002): 1) Dicer-like proteins (DCL) are RNase III enzymes that process long dsRNA and self-complementary hairpin RNA (hpRNA) precursors into 21-24 bp small interfering RNAs (siRNA) (Wilson and Doudna, 2013). These siRNAs bind with 2) argonaute proteins (AGO) to form the RNA-induced silencing complex (RISC). AGO selectively removes one strand of the siRNA duplex and uses the remaining strand to find messenger RNA (mRNA) with complementary sequence (Ketting, 2011). RISC mediates gene silencing by either cleavage of the mRNA that leads to rapid mRNA degradation, or by translational repression, whereby RISC stays bound to the mRNA and physically blocks ribosomes from translating protein (Wilson and Doudna, 2013). Finally, 3) RNA-dependent RNA polymerases (RdRp) are responsible for amplification of siRNA (Zhang and Ruvkun, 2012). Besides post-transcriptional regulation of gene expression, the known functions of RNAi include antiviral defense, transposon silencing, and epigenetic regulation (Ketting, 2011).

The knowledge that any gene's expression could be knocked down by complementary dsRNA let to rapid applications of this technology in both basic science and applied biotechnologies, including the development of pest and pathogen resistant plants. RNAi in plants directed at pathogens and pests is known as host-induced gene silencing (HIGS). The first example of transgenic plants expressing dsRNA that suppressed infection of a
pest was nematode-resistant *Arabidopsis thaliana* (Huang *et al*., 2006). Resistance to four major root knot nematode species was demonstrated in plants expressing a hpRNA that targeted parasitism gene 16D10 (Huang *et al*., 2006; Liley *et al*., 2007). HIGS has since been applied against numerous insects, viruses, and filamentous pathogens (fungi and oomycetes) (Rosa *et al*., 2018).

1.4.2 Current State and commercial examples

Very few HIGS plants are currently being used in agriculture. The majority of HIGS crops that have been approved for cultivation in the United States were developed in the 1990's prior to the discovery of RNAi. Virus resistant varieties of squash, potato, and papaya were developed by adding viral coat protein genes (Kreuze and Valkonen, 2017). The mechanism of resistance was later learned to be transgene-induced RNAi (Linbo and Falk, 2017). The most economically significant of these varieties was the Rainbow Papaya, which saved the Hawaiian papaya industry from devastation caused by the papaya ringspot virus and is still the predominant variety planted in Hawaii (Gonsalves 2006; Karst, 2022). More recently, HIGS has been commercialized in maize to control western corn rootworm (WCR, *Diabrotica virgifera*) (Head *et al*., 2017). The RNAi trait targets a vacuolar sorting protein (*Snf7*) and is intended to help overcome emerging WCR resistance to previously released transgenic varieties expressing insecticidal proteins from *Bacillus thuringiensis* (*Bt*). The latest varieties (SmartStax Pro) contain the RNAi trait stacked together with *Bt* insect resistance (cry proteins) and herbicide tolerance traits. Despite numerous applied studies in plant pathology, no other HIGS plants are widely grown. The reasons for limited use of HIGS in agriculture are unclear, but may be due to limited need, limited efficacy, or regulatory hurdles posed by the high costs of complying with EPA pesticidal registration requirements (Pierce *et al*., 2022).

1.4.3 Spray-induced gene silencing

Spray-induced gene silencing (SIGS) has emerged as another form of RNAi directed at pathogens and pests that is similar in effect to HIGS but does not require plant transformation (Rank and Koch, 2021). In SIGS, dsRNAs are applied as highly specific topical fungicides to plants and fruits (Niu *et al*., 2021). Early examples of SIGS included control of *Fusarium* head blight in barley (Koch *et al*., 2016) and control of grey mold
caused by Botrytis cinerea on fruits, vegetables, and flowers (Wang et al., 2016). SIGS has a few advantages over HIGS. Being transgene free, SIGS can be applied to protect non-transformable plants and this technology may be easier to commercialize as a pesticide due to high specificity and very narrow host range, without the regulatory burden associated with release of transgenic crops (Bramlett et al., 2020). SIGS against Colorado potato beetle is being commercialized by Greenlight Biosciences (Lexington, MA) and its planned release in the United States is currently undergoing federal review (Maxwell et al., 2018; De Schutter et al., 2022).

1.4.4 Technical aspects
HIGS transformation constructs are designed to produce either hpRNA or long dsRNA. The length of the dsRNA varies but is typically a few hundred bp per gene target. The simultaneous targeting of multiple genes (e.g., paralogs of essential genes) is often achieved by stacking dsRNA sequences together behind the same promoter. Longer dsRNA sequences result in a larger number of siRNA generated by DCL and a higher potential for silencing, while shorter sequences reduce the chances of non-target silencing (Höfle et al., 2020). Software design tools are available to select regions of the target mRNA without predicted off-target effects on host transcripts, and can also be used to avoid non-target effects on closely related species likely to be exposed to the dsRNA (Lück et al., 2019). These tools also identify regions of the target mRNA with the highest density of predicted efficient siRNA. Efficiency is determined by sequence-specific features that include accessibility of the target site based on predicted secondary structure, and base identities at both ends that promote loading onto AGO (Lück et al., 2019).

1.4.5 Fungal uptake of dsRNA
A critical success factor for either HIGS or SIGS against fungi is the uptake of dsRNA, the mechanisms of which are not well understood (Šečić and Kogel, 2021). Uptake of dsRNA requires passage through a hyphal cell wall made of chitin, polysaccharides, and glycoproteins, as well as a plasma membrane made up of lipids, proteins, and sterols (Riquelme et al., 2018). It’s thought that dsRNA uptake occurs predominantly at elongating hyphal tips, where the processes of exocytosis and endocytosis are most active.
(Riquelme et al., 2018). This hypothesis is supported by live cell imaging showing fluorescently labeled dsRNA localized into hyphal tips of the white mold pathogen *Sclerotinia sclerotiorum* (Wytinck et al., 2020). The same authors show that clathrin-mediated endocytosis (CME), a well conserved eukaryotic pathway (Kaksonen and Roux, 2018), was involved in uptake of dsRNA from *in vitro* culture.

In contrast to direct uptake, uptake from HIGS plants is thought to involve dsRNA released from plant cells in exosomes or extracellular vesicles (EVs) that then enter fungal cells through endocytosis (Cai et al., 2018). A competing theory suggests dsRNAs might also be exported from plant cells with RNA binding proteins (Karimi and Innes, 2022). With SIGS, uptake can occur either directly from dsRNA sprayed plant surfaces, or uptake can occur from plants similarly to HIGS (Rank and Koch, 2021). SIGS dsRNAs are thought to enter the plant apoplast through stomata, where they are then transferred to the symplast and then translocated through the vascular system (Biedenkopf et al., 2020). Depending on the feeding style of the fungus, uptake can occur from either the apoplast or the symplast (Biedenkopf et al., 2020).

Remarkably, fungal uptake of plant dsRNAs that induce RNAi also appears to occur naturally outside HIGS and SIGS treated plants (Cai et al., 2018; Jiao and Peng, 2018). For example, cotton plants transfer small RNAs to the vascular wilt fungus *Verticillium dahlia* to inhibit virulence genes (Zhang et al., 2016). Observations of cross-kingdom gene silencing (ckRNAi), both naturally and with HIGS/SIGS, suggest that dsRNA uptake mechanisms are present in many fungi, although uptake in most fungi has not been studied. However, a deficiency in uptake in *Zymoseptoria tritici* has been reported, either through direct means or via HIGS (Kettles et al., 2019).
1.5 Plant fungal microbiomes and the impacts of recombinant biotechnology

Plants are hosts to taxonomically and functionally diverse communities of fungi (Trivedi et al., 2020). Metagenomic approaches have greatly enriched our understanding of the diversity of fungi that colonize both plant surfaces (epiphytes) and within all available host tissues (endophytes) (Wilson, 2005; Hibbett et al., 2016). Most of these fungal species are commensal symbionts of unknown function (May, 2016); the majority of which are endophytic colonizers that do not cause disease (Saikkonen et al., 1998). Fungal microbiomes also include latent saprotrophs (Promputtha et al., 2010; Sun et al., 2011), which are mostly mutualists but also include the low abundance but very well studied category, pathogens (Carroll, 1998; May, 2016).

Microbiome composition is shaped by numerous factors that include host variation, the abiotic environment, species interactions, the dispersal of microbes across space, and the transmission of microbes between environments and hosts (Meyer et al., 2022, Bergmann and Leveau, 2022). For example, in leaves of deciduous trees, foliar communities reestablish each year as new leaves are flushed in spring (Barge, 2019). Communities initiate primarily by horizontal transfer, with colonization facilitated by the spread of inoculum through wind, rain splash, and biotic vectors (Rodriguez et al., 2009; Meyer et al., 2022).

Variation in host genotype and phenotype can act as filters to select for specific microbes (Wagner et al., 2016). In Populus, interspecific and intraspecific host genetics have been shown to influence microbiome composition (Bálint, 2013, Cregger et al., 2018; Barge, 2019; Leopold and Busby, 2020). For example, the genes involved in cuticle composition, chitin/flagellin detection, plant cell wall modification, and signal transduction have been shown to impact microbiome composition, and many of these genes are also involved in pathogen defense (Bressan et al., 2009; Balint-Kurti et al., 2010; Bodenhausen et al., 2014; Horton et al., 2014; Ritpitakphong et al., 2016). Given genetic influence over assembly, microbiomes have been described as extensions of plant phenotypes (Whitham, 2003); however, the influence of interspecific variation on microbiomes is typically overshadowed by environmental effects (Bálint, 2013; Wagner,
Furthermore, environment and host genetics can interact to modulate plant traits such as stomatal density and length that are consequential for colonization (Heitmann, 2022).

Plant-associated microbial communities can have important impacts on plant fitness and health (Hacquard and Schadt 2015). For instance, commonly occurring fungal leaf endophytes in poplar can alter the severity of disease caused by both biotrophic and hemibiotrophic foliar pathogens (Busby et al., 2013, 2016). Given the role of host genotype and phenotype in shaping microbial communities, it’s possible that perturbation of host genetics could affect plant-associated microbial communities, which in turn could have negative consequences for plant health. It’s therefore important to evaluate the potential risks to microbiomes that might be incurred by efforts to improve disease resistance in plants using transgenic or gene editing methods.

The methods employed for genetic transformation and gene editing typically cause significant physiological disruptions to plants. Repeated clonal propagation, particularly when it involves de novo callogenesis, followed by organogenesis or embryogenesis in the presence of plant growth hormones such as strong auxins, is well-known to generate aberrant phenotypic changes often referred to as somaclonal variation. Gene insertion also causes diverse mutations near to the insertion site that are known to include indels, duplications, and other rearrangements that can lead to transgene silencing and affect expression of nearby genes (Potrykus 1991, Birch 1997). Antibiotics are also typically used to enrich for modified cells, which can directly affect microorganism populations and induce random genome-wide mutations and epigenetic modifications (Bardini et al., 2003). Thus, in addition to the new properties imparted to plants by gene insertion or editing, the transformation process may affect microbiomes in the short and long term.

The effects of somaclonal variation on plant physiology might be difficult to measure directly. The evaluation of plant microbiomes using metabarcoding methods may be a more sensitive way too study somaclonal effects. Metabarcoding methods would also be
helpful in evaluating whether HIGS technologies imparted to control fungal pathogens are as specific to the targeted organism as expected.

1.6 References


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2. Transgenic *Populus trichocarpa* expressing double-stranded RNAs targeting *Sphaerulina musiva* CYP51 and DCL does not confer resistance to stem canker under greenhouse conditions

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2.1 Abstract

We explored host-induced gene silencing (HIGS) in transgenic poplar as a means to study and control *Sphaerulina musiva*, the cause of Septoria leaf spot and stem canker disease. Eighty-one independent lines (transgene insertion events) of HIGS transgenic poplars expressing double-stranded RNA (dsRNA) that targeted either or both *S. musiva CYP51* and *DCL* were developed and screened for resistance to stem canker disease in two greenhouse spray inoculation trials. We found no evidence of statistically significant differences in resistance between transgenic lines and wild-type controls (*p > 0.13*). The correlation between variation in greenhouse-expressed disease resistance and transgene expression was statistically significant among HIGS lines targeting *S. musiva DCL* (*r = -0.55, p = 0.03*), while no significant correlation was detected among HIGS lines targeting *S. musiva CYP51* (*r = -0.21, p = 0.34*). To evaluate the likelihood that HIGS or spray-induced gene silencing (SIGS) might be effective under some conditions, concurrent with greenhouse screening we conducted *in vitro* studies of: 1) *S. musiva*’s capacity for uptake of environmental dsRNA; 2) effects of *in vitro* silencing of *CYP51* and *DCL* on fungal growth and target transcript abundance; and 3) persistence of dsRNA in culture. No uptake of fluorescently tagged dsRNA was detected *in vitro* with confocal live imaging. In dsRNA-treated cultures, no fungal growth inhibition was detected, and RNA was rapidly degraded in the culture medium by a factor susceptible to proteinase K digestion. Of five target transcripts tested after dsRNA treatment, only one had statistically impaired expression, suggesting there was a low level of selective RNA uptake. Our results suggest that HIGS and SIGS are unlikely to be effective control measures for this disease.

2.2 Introduction

2.2.1 Septoria leaf spot and stem canker disease in poplar

Hybrid poplars are important sources of fiber and lumber (Dickmann *et al.*, 2001). Across North America, productivity of short rotation plantations is threatened by Septoria leaf spot and stem canker disease caused by the ascomycete fungus *Sphaerulina musiva* (syn. *Septoria musiva*) (Ostry and McNabb, 1985; Newcombe and Ostry, 2001; Feau *et al.*,
S. musiva is a hemibiotroph that infects both foliar and woody tissues through natural openings such as stomata, lenticels, and petiole junctions (Weiland and Stanosz, 2007; Qin and LeBoldus, 2014). Colonization by fungal hyphae is restricted to the apoplast where subsequent transition to necrotrophy gives rise to canker and leaf spot symptoms (Abraham et al., 2019). While leaf spots lead to premature defoliation and reduced photosynthetic capacity, stem cankers pose the more significant threat to plantations as cankers girdling the vasculature increase the risk of wind-induced stem breakage and can kill trees outright (Bier, 1939; Waterman, 1954; Ostry and McNabb, 1985; Feau et al., 2010). Several studies have noted devastation of hybrid plantations by Septoria canker (Ostry and McNabb, 1985; Lo et al., 1995; Weiland et al., 2003; LeBoldus et al., 2009). For example, in a mixed-hybrid Populus plantation in Michigan, 86% of trees had Septoria cankers five years after planting, and 69% had broken tops two years later (Ostry et al., 1989). This ultimately led to plantation failure as there were too few trees likely to survive till harvest age to remain economically viable. Western cottonwood (Populus trichocarpa Torr. and Gray) is a naive host to S. musiva and generally lacks resistance (Muchero et al., 2018). Populus hybrids with P. trichocarpa parents carry dominant susceptibility and as a consequence the progeny of many desirable P. trichocarpa crosses cannot be grown in areas with high disease pressure (Newcombe and Ostry, 2001). Genetic resistance has long been considered the best way to manage this disease; however, breeding programs can take several years to develop resistant cultivars (Ostry, 1987a). Transgenic approaches leading to heritable resistance have the potential to be developed more quickly, and these efforts can complement conventional approaches with novel traits introgressed into site-adapted elite cultivars (Forest Health and Biotechnology, 2019; Newhouse and Powell, 2021).

2.2.2 HIGS
Host-induced gene silencing (HIGS) has become a commonly studied method for engineering plant protection against pathogens and pests using genetic engineering methods (Koch and Wassenegger, 2021a). HIGS takes advantage of widely conserved RNA interference (RNAi) mechanisms present in most eukaryotes (Shabalina and Koonin, 2008). RNAi refers to the process of post-transcriptional gene silencing (PTGS) of specific messenger RNA (mRNA) that is mediated by small RNA (sRNA) with
complementary sequences (Hammond et al., 2001). In HIGS, the sequence of the silencing RNA is carefully selected to determine which genes can be effectively silenced. The sRNAs that trigger RNAi are double stranded RNA (dsRNA) and are processed by dicer-like proteins (DCL) into small interfering RNAs (siRNAs) that are ~ 21-24 bp in length. siRNAs then complex with argonaute proteins (AGO) that selectively remove one strand and use the other to form an RNA-induced silencing complex (RISC). RISC then silences (i.e., knocks-down) mRNA with complementary sequence to the siRNA by transcript cleavage or translational inhibition. RNAi also includes processes of transcriptional gene silencing (TGS) whereby siRNA and AGO guide a DNA methyltransferase as part of an RNA-induced transcriptional silencing complex (RITS) to epigenetically silence transcription of genes with complementary sequences (Bhattacharjee et al., 2019). Most HIGS studies focus on PTGS though it's unclear if PTGS contributes more to target gene silencing in HIGS applications than does TGS (Karimi and Innes, 2022).

Since the term HIGS was coined by Nowara et al. (2010), the method has been applied against viruses, insects, and nematodes in diverse host species (Koch and Wassenegger, 2021a). An early and influential example of HIGS was reported by Koch et al. (2013). They demonstrated resistance to Fusarium graminearum in both barley and Arabidopsis by using HIGS to knock-down the same genes targeted by azole fungicides. By expressing dsRNA complementary to all three paralogs of F. graminearum cytochrome P450 lanosterol C14α-demethylase genes (CYP51), they demonstrated the potential of HIGS to limit dependence on fungicides. In another study, Wang et al. (2016) demonstrated that B. cinerea RNAi genes (DCL1 and DCL2) were also effective HIGS targets. Dicer genes appear to be effective HIGS targets in B. cinerea because they are essential for the biogenesis of siRNA effectors that are trafficked into plants to silence host immunity genes (Weiberg et al., 2013; Wang et al., 2016a). This phenomenon is known as cross-kingdom RNAi (ckRNAi) and has been observed in the opposite direction with plants using siRNA to silence fungal genes (Cai et al., 2018a). Observations of ckRNAi and HIGS both demonstrate that at least some fungi carry mechanisms for uptake of plant-derived dsRNA.
RNAi-mediated control of pest and pathogens has also been demonstrated by spraying dsRNAs directly onto plants (Koch et al., 2016; Wang et al., 2016a; Rank and Koch, 2021a). Known as spray-induced gene silencing (SIGS), this strategy can be as effective as HIGS and has the potential to offer the most pathogen-specific fungicides ever developed (Koch et al., 2019; Hough et al., 2022). SIGS may eventually be applied more than HIGS as it skirts the need for host transformation and public acceptance of genetically modified organisms. SIGS studies have demonstrated additional routes by which fungi take up dsRNA. In addition to uptake from HIGS plants, some fungi can also take dsRNA directly from the environment (Wang et al., 2016a; McLoughlin et al., 2018; Qiao et al., 2021). With SIGS, it is thought that fungi take up dsRNA both from the leaf surface, and from plant tissues after dsRNA has first been absorbed and translocated within the plant (Niu et al., 2021a). The mechanisms of fungal uptake continue to be studied (Karimi and Innes, 2022).

While RNAi is nearly ubiquitous in fungi (Nakayashiki et al., 2006), the ability to take up dsRNA—either from the host or the environment—may be much less common. Kettles et al. (2019) have shown that the wheat pathogen *Zymoseptoria tritici* may not be amenable to HIGS and SIGS. *In vitro* RNAi and HIGS against essential *Z. tritici* genes was inefficient at reducing fungal growth and virulence respectively. They also showed a lack of uptake of fluorescently tagged dsRNA in culture using live imaging. In a similar approach, Qiao et al. (2021) found variable environmental dsRNA uptake efficiencies among five fungi. When HIGS was tried against the same fungi, the only species whose virulence was not attenuated by HIGS was the one fungus with the least efficient uptake *in vitro* (*Colletotrichum gloeosporioides*). It therefore appears that dsRNA uptake mechanisms critical for HIGS and SIGS are not present in all fungi.

2.2.3 Study goals

The goal of this study was to see if HIGS-based resistance to *S. musiva* could be engineered in *P. trichocarpa*, including to analyze the potential for RNA uptake. Gene targets *CYP51* and *DCL* were the *S. musiva* homologs of gene targets found effective with HIGS against *B. cinerea* and *F. graminearum* (Koch et al., 2013; Wang et al.,
Existing transcriptomics for *S. musiva* (Muchero et al., 2018) show that *S. musiva* core RNAi genes (dicer-like, argonaute, and RNA-dependent RNA polymerase) are actively transcribed during early stages of infection and suggest functioning RNAi. We report no resistance in greenhouse studies and inefficient uptake *in vitro*. To our knowledge, this is the first report of HIGS applied in an angiosperm forest species against a fungal pathogen.

### 2.3 Materials and Methods

#### 2.3.1 ID of gene target homologs

*S. musiva* has one cytochrome P450 lanosterol C-α-demethylase gene (*CYP51*, SEPMUDRAFT_151286) that shares 60.3% identity with *Fusarium graminearum CYP51-B* (FGSG_01000) targeted by Koch et al. (2013). Unlike *F. graminearum* which has three *CYP51* copies (Liu et al., 2011), *S. musiva* has only one clear homolog. Without a known phenotype for *CYP51* in *S. musiva*, we opted to target two related genes that could possibly provide functional redundancy. The protein sequence for *S. musiva CYP51* was used to identify two homologous genes based on analysis of shared domains and structural predictions in Phyr2, and phylogenetic trees built from a jackHMMER search. Homologs *CYP6A1* (SEPMUDRAFT_149948) and *CYP61* (SEPMUDRAFT_147259) were also targeted by our RNAi constructs.

*S. musiva* has two dicer-like (*DCL*) copies: *DCL-1* (SEPMUDRAFT_72068) shares 34.0% identity with *Botrytis cinerea DCL-1* (BCIN_12g06230), and *S. musiva DCL-2* (SEPMUDRAFT_136183) shares 32.4% identity with *B. cinerea DCL-2* (BCIN_14g03910)—both of which were targeted by Wang et al. (2016). A similar search for homologs of *DCL1* and *DCL2* (SEPMUDRAFT_72068, SEPMUDRAFT_136183) failed to identify additional genes suspected to encode functional redundancy.

To ensure the relevance of target genes during infection, the expression of all five *S. musiva* gene targets were checked against published transcriptomes (Dhillon et al., 2015). The protein sequences of these *S. musiva* gene targets, and those of their homologs
targeted in other HIGS studies, were aligned using MAFFT (Katoh and Standley, 2013) and the conserved amino acid sites were used to generate neighbor-joining trees (Figure S2.1).

2.3.2 RNAi constructs
RNAi constructs for in vitro RNAi and HIGS transgenics were designed using siRNA Finder version siFi21_1.2.3-0008 (Lück et al., 2019). Gene target fragments—ranging in size between 251-315 bp—from within the target mRNA coding sequence were selected based on both the predicted density of high efficiency siRNAs (Lück et al., 2019), and lack of off-target silencing against Populus trichocarpa v3.1 transcripts. Stacked RNAi constructs for like gene targets were synthesized and inserted into backbones p9U10-RNAi and p6U10-RNAi by DNA Cloning Service (Hamburg, Germany). p9U10-RNAi-Sm-CYP51 targets S. musiva CYP51 and two of its homologs while p6U10-RNAi-Sm-DCL targets both S. musiva DCL genes. p9U10-RNAi-GUS targets E. coli beta-glucuronidase (GUS) and serves as a non-specific double stranded RNA (dsRNA) control (Figure S2.2).

2.3.3 S. musiva culture
Sphaerulina musiva isolate MN-14 (Dunnell and LeBoldus, 2017) was used for all experiments due to its prolific sporulation in culture and strong symptom development in Populus trichocarpa clone SLMB-28-1. MN-14 was routinely cultured on KV-8 agar (2 g calcium carbonate, 20 g agar, 820 mL deionized water, and 180 mL V-8 vegetable juice (Campbell Soup Company, Camden, NJ, USA)) under continuous lighting for one to two weeks to induce sporulation. Conidia were harvested and quantified by flooding plates with sterile water, scraping mycelia, and counting cells with a haemocytometer. This isolate was re-isolated from stem cankers in between greenhouse experiments to maintain its profuse sporulation in culture, which would otherwise diminish over repeated subculturing.
2.3.4 In vitro RNAi

2.3.4.1 dsRNA
Synthetic double stranded RNAs (dsRNA) used to assay target gene knock-down, growth inhibition, and dsRNA stability were sourced from Greenlight Biosciences (Lexington, MA) (Figure S2.3). *Sm-CYP51* dsRNA (891 bp), *Sm-DCL* dsRNA (645 bp), and *GUS*-dsRNA (594 bp) are identical in sequence to dsRNAs produced by our RNAi constructs (Figure S2.2) minus the restriction sites added between stacked gene fragments, and the addition of 15 bp ITS sequences at both ends.

2.3.4.2 Growth inhibition
To quantify inhibitory effects of fungicide and dsRNAs on fungal growth, we used the *in vitro* assay described by Koch *et al.* (2013). Because azole fungicides interfere with CYP51, we treated *S. musiva* with increasing concentrations of Tebuconazole (Orius® 20AQ, Makhteshim Agan of North America) to suggest whether RNAi against *CYP51* might also work to limit growth. *S. musiva* cells treated with Tebuconazole or dsRNAs *Sm-CYP51, Sm-DCL*, or *GUS* control were assayed for growth inhibition in microliter plates using optical density (OD600) as a proxy for growth. Wells containing $10^5$ conidia, dsRNA (250, 500 or 1000 ng), and 0.8% w/v potato dextrose broth (PDB) in a total volume of 200 µl were cultured at room temperature in the dark for 48 hours before OD600 was measured on a Tecan Spark 10M microplate reader in technical triplicate. Amounts of dsRNA were consistent with those previously reported (Koch *et al.*, 2013; Wang *et al.*, 2016a; Kettles *et al.*, 2019). Plate readings at time zero were used as blanks to subtract background absorbance. Technical replicate OD600 readings were averaged and normalized to average OD600 values for growth of untreated cell controls. One-tailed t-tests were used to test for reductions in mean response relative to the control. *Post hoc* power analysis using observed response variation was used to suggest the detectable effect size (one-tailed t-test, n=3, alpha= 0.05 and power =0.80). This experiment was repeated three times.

2.3.4.4 dsRNA longevity in culture
In microwell plates, $10^5$ *S. musiva* conidia were cultured in 0.8% (w/v) potato dextrose broth containing 1 µg *GUS*-dsRNA in a total volume of 100 µl. Cultures were incubated
at room temperature in the dark. Supernatants were harvested every 12 hours for 72 hours and stored at -80°C until all samples were collected. Supernatants from the entire 72-hour period were then thawed, combined with 10 µl gel loading buffer, and run through large format 1% agarose gels (wells ~150 µl, 100 v for 50 min) to make qualitative evaluations of band size and intensity—compared to sterile control wells that received only broth and dsRNA. An 8-day old culture of *S. musiva* in 0.8% potato dextrose broth was filter sterilized (0.2 µm) and used in place of fungal spores in the above assay. Sterility of the filtrate was checked on KV-8 agar. The same culture filtrate treated with proteinase K (New England Biolabs #P8107S) (30 units/ml filtrate, 37° for 1 hour) was also used in place of fungal spores in the above assay.

2.3.4.5 Knock-down of fungal gene targets
*S. musiva* was cultured in KV8 broth (pH 7) with 2x10^4 conidia/ml and 100 ng/µl dsRNA in flasks shaken at 200 RPM in the dark at room temperature. Cultures were treated with either *Sm-CYP51* dsRNA, *Sm-DCL* dsRNA, or *GUS* control dsRNA. After 24, 36, and 48 hours, mycelia were vacuum filtered, rinsed with sterile water, added to 2 ml lysing tubes (MP bio lysing matrix I, #116918050-CF), frozen on liquid nitrogen, dry homogenized for 20 seconds at 6 m/s (Savant FastPrep FP120 Cell Disruptor), returned to liquid nitrogen for 2 min, and dry ground two additional times. Total RNA extraction with Trizol, DNase treatment, reverse transcription, and qPCR were performed as noted in section 2.3.8. Relative expression was normalized to fungal reference gene actin (SEPMUDRAFT_147624). Two-tailed t-tests were used to test for differences in mean dCts relative to the control. Primers used for relative expression targeted regions of transcripts that did not overlap with dsRNA constructs, therefore any residual dsRNA treatment captured during total RNA isolation and present in cDNA libraries could not be amplified during qPCR (Table S2.1). Independent experiments for each timepoint were repeated at least three times.

2.3.4.6 Assessment of tagged dsRNA uptake
The ability of *S. musiva* isolate MN-14 to uptake exogenous siRNA was assessed using BLOCK-iT™ Alexa Fluor™ Red Fluorescent Control (Invitrogen #14750100). For uptake of long dsRNA, a 741 bp dsRNA complementary to enhanced green fluorescent
protein (eGFP) was transcribed using cy3-UTP (APExBIO #B8330) along with HiScribe® T7 High Yield RNA Synthesis Kit (New England Biolabs #E2040S). Microscope slides were coated with 1% agar containing 0.1% (w/v) potato dextrose broth (BD Difco #254920) and prepared with simultaneous and overlapping applications of 500 conidia applied in 5 µl, and either 500 ng dsRNA or 500 ng siRNA in 5 µl. Controls were similarly treated with either 5 µl water or 20 µM cy3-UTP. Slides were incubated in a moist chamber in the dark at room temperature for either 12, 24, or 48 hours and then treated with 75 U MNase at 37 °C for 30 min (New England Biolabs # M0247S) before viewing on a ZEISS LSM 780 NLO confocal microscope (Confocal Microscopy Facility of the Center for Quantitative Life Sciences at Oregon State University). Botrytis cinerea spores treated as above served as a positive control for dsRNA uptake (Wang et al., 2016). The B. cinerea isolate used in this study was isolated from P. trichocarpa leaves in Corvallis, OR and identified by its ITS2 sequence. Laser intensity for red fluorescence (excitation/emission 555/565 nm) was first set with the cy3-dsRNA treated B. cinerea cells and the same settings were used for all other treatments. Experiments at the 12-hour timepoint were repeated three times.

2.3.5 HIGS transformations
S. musiva-susceptible Populus trichocarpa genotype SLMB-28-1 (T61) (Muchero et al., 2018) was transformed with the constructs above (Figure S2.2a) using standard Agrobacterium methods (Ma et al., 2004; Song et al., 2006; Li et al., 2017). Leaf and stem explants were co-cultivated with Agrobacterium strain AGL1 at a concentration of 0.6-0.8 optical density. Shoots forming under hygromycin and or kanamycin selection were PCR confirmed for the full-length dsRNA transgenes. A total of 81 HIGS events were generated including 39 p9U10-RNAi-CYP51 events, 30 p6U10-RNAi-DCL events, 12 events co-transformed with both p9U10-RNAi-CYP51 and p6U10-RNAi-DCL, and six p9U10-RNAi-GUS control events.

2.3.6 Poplar culture
Transgenic events and WT controls were maintained and replicated in vitro on woody plant media with rooting hormone (WPM-RT). Rooted plants were acclimated to soil (SunGro Sunshine #4) in 2¼ x 3¼ inch pots (Anderson #1683) placed in sealed 1-gallon
plastic bags with an 18-hour photoperiod for three weeks in a headhouse. Plants were acclimated by first opening plastic bags for one week, then pots were transferred into translucent plastic tubs with slightly opened lids in a greenhouse for two additional weeks. Lids were gradually removed over the first week. Acclimated greenhouse plants were potted in 2.5 x 10-inch Deepot cells. Plants were fertilized weekly with liquid 20-20-20 (Harrell's # 880105-VI) at 500 ppm N. Side shoots were trimmed to encourage growth of the main stem. Plants received an 18-hour photoperiod. Fertilization was discontinued at the start of the inoculation experiments.

2.3.7 Greenhouse resistance screening
Trees were inoculated by spraying all stem and leaf surfaces to run-off with conidia suspended in sterile water using a spray bottle. Inoculated trees were initially covered in plastic bags and incubated in the dark for 48 hours to maintain relative humidity and encourage spore germination. The germination rate was quantified by counting spores plated on water agar for 12 hours. Symptoms developed over three weeks. Disease severity was quantified as the mean number of stem cankers formed 3 weeks post-inoculation (WPI) normalized by height in cm at time of inoculation.

2.3.7.1 Resistance screen 2020
The first experiment containing the initial transformants was challenged with a 5x10^5 conidia/ml inoculum. Line (event) level replication was five ramets and the experiment contained a total of 129 ramets that survived soil acclimation. All T61 trees were allowed to reach an average height of 48.7±8.86 cm before inoculation. Resistant hybrids 353 (tremula x tremuloides) and 717 (tremula x alba) were included as positive controls for resistance. One stunted ramet (< 21 cm) was removed from the analysis.

2.3.7.2 Inoculum optimization
To determine the lowest concentration of inoculum that could reliably produce stem cankers on all individuals within a treatment group, WT-T61 (SLMB-28-1) plants were inoculated with spore suspensions ranging ten-fold from 10^2 to 10^6 conidia/ml. Each group contained 5 ramets. The average tree height at the time of inoculation was 79.0±7.86 cm.
2.3.7.3 Resistance screen 2022
HIGS lines were screened as in the 2020 trial with three modifications: (1) the inoculum dose was decreased to $10^5$ conidia/ml to avoid overwhelming potential resistance, (2) ramet level replication was increased to raise experimental power, (3) events were selected to focus on four of the highest HIGS transgene expression events and two low expression events for each *S. musiva* targeting construct type (*Sm-CYP51*, *Sm-DCL*, and *Sm-CYP51 + Sm-DCL*). Trees were allowed to reach an average height of 29.22±5.06 cm prior to inoculation. Stunted ramets (< 19 cm) were removed from the analysis.

2.3.7.4 Statistical analysis
Analysis of variance (ANOVA) was used to test for effects of construct and event on disease severity (cankers/cm) with starting height included as a covariate. Because cankers form on all sides of a stem, and stem height and stem volume are not linearly related, inclusion of starting height as a covariate appeared to help correct for the limitations of normalization by height alone. Dunnett’s test, a multiple comparisons-corrected test for the case of comparing all treatments to a control, was used to compare all experimental treatments to the wild type. As discussed under results, post-hoc power analysis of the 2020 experiment was used to inform the 2022 experiments.

2.3.8 Transgene expression
Relative HIGS transgene expression was measured across all HIGS transgenic events to: (1) ensure transgenes were expressed in most lines; (2) identify the highest expressing lines; (3) to focus continued resistance screening efforts on these highest expressing lines; and (4) to alleviate concerns of transgene silencing that might have prevented target gene RNAi. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was used to measure relative expression of both HIGS and marker gene transcripts produced by our RNAi constructs. Total RNA was extracted from combined leaf and stem tissues of single ramets in micropropagated culture using Trizol reagent (Invitrogen #15596) following the manufacturer’s instructions. A high salt buffer (0.8 M sodium citrate and 1.2 M NaCl) was used 1:1 with isopropanol during RNA precipitation to aid in removal of plant polysaccharides. Total RNA preps were treated with Turbo DNase (Invitrogen #AM2238). Total RNA was reverse transcribed with a mix of both oligo-dT and random
primers using iScript cDNA synthesis kit (Biorad#1708891) with 500 ng total RNA in 10 µl reactions. Relative transcript abundance was quantified using PowerUp™ SYBR™ Green Master Mix (Applied Biosystems #A25742) using 10 ng cDNA as template in 10 µl duplicate reactions with forward and reverse primers at 500 nM each (Table S2.1). Expression of HIGS and marker transcripts were normalized to host reference gene elongation factor 1-beta (Potri.009G018600). Relative expression was calculated as $2^{-\Delta \Delta Ct}$ where $\Delta Ct$ is difference in cycle thresholds ($Ct_{\text{gene of interest}} - Ct_{\text{reference}}$). Pearson's product-moment correlation coefficients were calculated to test for negative correlations of HIGS transgene expression and disease severity with p-values representing one-tailed tests.

2.4 Results

2.4.1 S. musiva is sensitive to azole fungicide
The minimum inhibitory concentration (MIC) of tebuconazole that limited visible growth was 200 nM (Figure S2.4). No growth was detected at 400 nM. These concentrations are three orders of magnitude below the recommended dilute spray rate for control of foliar leaf spot pathogens (4.3 oz/100 gal or ~200 µM tebuconazole).

2.4.2 In vitro RNAi targeting CYP51 and DCL genes does not inhibit S. musiva growth
To determine whether in vitro silencing of CYP51 and DCL would impair growth, germinating conidia were treated with 891 bp Sm-CYP51 dsRNA and 645 bp Sm-DCL dsRNA respectively in microwell culture. A 594 bp GUS-specific dsRNA served as a negative control. Growth of cells treated with either Sm-CYP51 dsRNA or Sm-DCL dsRNA at any of three doses (250, 500, or 1000 ng) was not statistically different than the control treatments (p > 0.25) (Figure S2.5). The standard deviations between replicate responses (optical density relative to untreated cells) within each of nine treatment groups had an average of 23.0%. A post hoc power analysis using this variation suggested an effect size of 70.6% (growth reduction) was necessary to detect a significant difference at (alpha = 0.05).

2.4.3 Rapid degradation of dsRNA in both culture and culture filtrate
To measure the duration of time that full length dsRNAs remained intact and in solution under the in vitro conditions used above, supernatants from GUS-dsRNA treated
microwell cultures were collected over 72 hours. Band size and intensities were qualitatively compared to control wells that received only broth and dsRNA. After 24 hours, no dsRNA band was visible in supernatant from treated culture while dsRNA bands from control wells remained intact over 72 hours (Figure S2.6a). The disappearance of dsRNA over time could be explained by either extracellular dsRNA degradation, and or fungal uptake. Therefore, dsRNA was also treated with the filtrate of S. musiva culture to test for secretion of RNase. After culture filtrate and dsRNA were incubated together for 8 hours, dsRNA was no longer visible on a gel (Figure S2.6b). To establish whether this result was due to a secreted proteinaceous nuclease, we also incubated dsRNA with culture filtrate treated with proteinase K (Figure S2.6c). Contrary to the results of filtrate treated dsRNA, dsRNA treated with proteinase K treated filtrate persisted over 72 hours.

2.4.4 In vitro RNAi against Sphaerulina musiva results in down-regulation of one of five gene targets

To determine whether target transcripts were silenced in vitro, total RNA was extracted from dsRNA treated cultures over 48 hours, and transcript abundance was measured using RT-qPCR. Having previously observed the rapid disappearance of dsRNA treatments from solution within 24 hours (Figure S2.6a), the dsRNA concentration in this experiment was increased 10-fold to ensure that dsRNA remained available for the duration of the experiment. Analysis of dsRNA remaining in culture supernatant after 48 hours on agarose gels had dsRNA bands of equal intensity to dsRNA bands of the 0-hour supernatant and suggested dsRNA was available for uptake throughout the duration of the experiment (gel not shown).

Of the three transcripts targeted by Sm-CYP51-dsRNA, no silencing was observed at 24, 36, or 48 hours (Figure 2.1a). Of the two transcripts targeted by DCL-dsRNA, only DCL1 expression was silenced (Figure 2.1b). Knock-down was observed for DCL1 at all three time points and ranged from 92.4% at 24 hours to 79.4% at 48 hours (DCL1 vs. control difference in mean dCt = 3.7124-H, 3.5936-H, 2.2848-H). In contrast, the average difference in mean dCt for the other four targets at all three timepoints was -0.07±0.72 standard deviations. The average reference gene Ct was 19.3±1.0 cycles.
Figure 2.1 *In vitro* knockdown of *S. musiva* gene targets

dsRNAs treatments targeted either a, *CYP51* homologs (cyp51) or b *DCL* homologs (dcl), with a GUS non-specific dsRNA serving as the (control) treatment. The Y-axis shows Delta Ct (dCt) for all five gene targets. Each dot within a timepoint represents an independent experiment. Red asterisks highlight significant downregulation with P-values for two-tailed t-tests shown above the brackets.
2.4.5 No detection of uptake of fluorescent dsRNA using live cell imaging

To investigate the efficiency of dsRNA uptake by *S. musiva in vitro*, we used fluorescently tagged dsRNAs with confocal microscopy (Galli *et al.*, 2020; Hamby *et al.*, 2020). Autofluorescence at green wavelengths was observed in germinating *S. musiva* conidia that precluded use of fluorescein in tagged dsRNA uptake experiments. Conidia were germinated on agar coated slides containing either long 741 bp cy3-labeled dsRNA complementary to *eGFP*, Alexa Fluor 555-labeled siRNA with random sequence not complementary to any known gene, or controls cy3-UTP or water. After 12 hours, slides were treated with micrococcal nuclease to degrade extracellular dsRNA and imaged using a confocal microscope. *Botrytis cinerea* was used as a positive control for uptake as its capacity for environmental dsRNA uptake has been well established (Wang *et al.*, 2016a; Kettles *et al.*, 2019; Qiao *et al.*, 2021). As expected, fluorescence was detected in germinating *B. cinerea* spores treated with both long dsRNA and siRNA after 12 hours (Figure 2.2). *S. musiva* spores however had no fluorescent signal detected despite a 100% germination rate at 12 hours. Similar results were also observed at 24 and 48 hours (not pictured).

![Figure 2.2 In vitro uptake of red fluorescent dsRNA](image-url)
Confocal imaging of germinating *S. musiva* and *B. cinerea* conidia are shown after treatment with either cy3-eGFP-dsRNA, BLOCK-iT™ siRNA (Alexa Fluor™), or controls cy3-UTP or water (not pictured), all followed by MNase treatment after 12 hours. Similar results were observed at 24 and 48 hours (not pictured).

2.4.6 Inability to detect enhanced resistance in first greenhouse inoculation trial (2020)
To phenotype initial HIGS transformants for resistance to *S. musiva* stem canker, we inoculated trees in a greenhouse experiment (Figure 2.3). No statistically significant differences in mean height between constructs were detected, nor between events within constructs, that would suggest deleterious transgene or insertion effects. The mean disease severity was 0.469±0.204 cankers/cm, and all plants developed at least 2 cankers. Inoculum viability was 100% for this and all other greenhouse trials.

We divided canker counts by tree height to normalize counts for expected differences between taller and shorter trees. Despite this normalization, shorter trees tended to have lower normalized counts. When modeling for construct and event effects on disease severity, starting height was included as a covariate (p < 0.02).

We failed to reject the null hypothesis that construct had no effect on disease severity (ANOVA, $F_{(3, 100)} = 1.73, p = 0.165$). Similarly, we failed to reject the null hypothesis that event had no effect on disease severity (ANOVA, $F_{(21, 82)} = 1.31, p = 0.191$). As expected, the positive controls for resistance (hybrids 353 and 717) both had lower disease severity than WT-T61 (Figure S2.7) (ANOVA, $F_{(3, 100)} = 33.3, p = 3.10 \times 10^{-7}$). The effect sizes for the positive controls were a 72.7% (353) and a 75.3% (717) reduction in disease severity relative to WT. *Post-hoc* power analysis resulted in an 80% power to detect a 66.4% (delta = 0.411 cankers/cm) reduction in the mean disease severity between HIGS events and WT-T61 (Figure S2.8).
2.4.7 Inoculum optimization

The absence of resistance among initial transformants (Figure 2.3) prompted consideration of lower doses of inoculum that could still elicit reliable canker symptom development without overwhelming potential resistance. The dose response in Figure S2.9 was used to select $10^5$ conidia/ml as the inoculum concentration for subsequent trials.
2.4.8 Transgene expression

A wide range of HIGS transgene expression was observed among events of the same construct (Figures 2.4, 2.5). For events of p9U10-RNAi-Sm-CYP51, expression ranged from 0.84±0.14 times that of the elf1-β reference cDNA to undetectable (Figure 2.4). For events of p9U10-RNAi-Sm-DCL, expression ranged from 2.96±0.96 times that of reference to undetectable (Figure 2.5).

Relative expression was also measured for marker genes contained on the same transfer DNA (tDNA) under different promoters (Figure 2.4 and Figure 2.5). Unexpectedly, weak correlations were observed between relative expression of HIGS transgenes and the marker gene among p9U10-RNAi-Sm-CYP51 events (r = 0.16, p = 0.12) while the correlation to marker gene expression among p6U10-RNAi-Sm-DCL events was much stronger (r = -0.56, p < 0.001) (Figure S2.10). Several events with some of the highest marker expression had little to no HIGS transgene expression detected (e.g., cyp-47, Figure 2.4).
Figure 2.4 Transgene expression across p9U10-RNAi-Sm-CYP51 events
a Relative expression of HIGS transgene and b selectable marker. Each bar represents the average of two biological replicates with error bars denoting standard error. Events labeled cyp are single transformants while c+d were co-transformed with p6U10-RNAi-Sm-DCL.

Figure 2.5 Transgene expression across p6U10-RNAi-Sm-DCL events
a Relative expression of HIGS transgene and b selectable marker. Each bar represents the average of two biological replicates with error bars denoting standard error. Events labeled DCL are single transformants while c+d were co-transformed with p9U10-RNAi-Sm-CYP51.

2.4.9 HIGS resistance not detected in 2022 greenhouse trial.
A second greenhouse experiment was used to screen high HIGS transgene expression events and controls for resistance (Figure 2.6). The reduced inoculum concentration used in this trial resulted in a reduced mean disease response compared to the 2020 experiment. The overall disease severity was 0.186±0.116 cankers/cm. There were 12 plants out of 399 that failed to develop cankers. The 2022 coefficient of variation (CV) was 62.4 while the 2020 experiment CV was 43.7. Despite the increase in CV in the 2022
experiment, the sensitivity of the experiment was improved over the 2020 experiment. 

*Post hoc* power analysis given a mean sample size of n = 16 resulted in 80% power to
detect a 50.9% reduction (delta = 0.119 cankers/cm) in mean disease severity relative to
the observed mean for WT (Figure S2.11). The detectable effect size was 23.3% smaller
than the effect size for the 2020 experiment (66.4%).

Again, starting height was a significant covariate when modeling construct and event
effects on disease severity (p > 0.001). We failed to detect a statistically significant effect
of construct on canker density (ANOVA, F(5, 392) = 1.86, p = 0.101). However, there was
strong evidence that events differed in this experiment (ANOVA, F(24, 373) = 1.99, p =
4.21x10^{-3}). Nonetheless, multiple comparisons against WT failed to identify any single
events that were significantly different in canker density (p > 0.130 for all contrasts)
(Figure S2.10).
Figure 2.6 Resistance phenotypes of HIGS lines screened in 2022 greenhouse experiment
Bars show mean number of stem cankers formed three weeks post inoculation, normalized by plant height at time of inoculation. Raw data is overlaid as dots. Unique transgenic events and controls are listed on the x-axis and color is used to show construct and control groups. For constructs cyp, dcl, and cyp+dcl, events are ordered left to right from high to low expression of HIGS transgenes as measured by RT-qPCR. WT-T61 (*P. trichocarpa* SLMB-28-1) is the susceptible transformation background. NTE = non-transgenic escape with line numbers representing independent regenerated shoots that escaped selection. Constructs cyp (p9U10-RNAi-Sm-CYP51) and dcl (p6U10RNAi-Sm-DCL) target *S. musiva* genes while GUS (p9U10-RNAi-GUS) is a non-specific RNAi control.

2.4.10 Significant correlation between greenhouse resistance and expression of HIGS transgenes targeting *S. musiva* DCL
Disease severity and HIGS transgene expression among p9U10-Sm-CYP51 events was weakly correlated in both the 2020 trial \((r = -0.17, p = 0.30)\) and the 2022 trial \((r = -0.21, p = 0.34)\) (Figure 2.7a). When including co-transformed events in the 2022 trial, the correlation was stronger but not significant \((r = -0.41, p = 0.09)\). The correlation between disease severity and HIGS transgene expression among p6U10-Sm-DCL events was strong \((r = -0.48, p = 0.17)\) (Figure 2.7b). When including co-transformed events, the correlation was stronger and significant \((r = -0.55, p = 0.03)\).
Figure 2.7 Correlation of greenhouse resistance phenotypes with HIGS transgene expression

Mean resistance phenotypes (cankers/cm) from greenhouse inoculations in relation to mean relative expression of HIGS transgenes. Dots are unique transgenic events and color shows which *S. musiva* targeting construct(s) they contain. Pearson's correlation coefficients and p-values are given for both single transformants only, and for combined single and co-transformed events (blue). A Correlation among p9U10-Sm-CYP51 events in two separate greenhouse trials. B Correlation among p6U10-Sm-DCL events in 2022 trial.
2.5 Discussion

For HIGS to potentially inhibit a fungal disease, it requires selection of target gene(s) that are lethal or essential for virulence, effective RNAi trigger selection (dsRNA sequence and length), and a suitable expression vector. It also requires host expression of a relevant dsRNA dose, host transfer and fungal uptake of dsRNA, an active fungal RNAi system, and sufficient silencing of target transcripts. When HIGS fails to inhibit disease severity, any one of these factors could be limiting. Overall, our results point to limited dsRNA uptake efficiency and variable RNAi trigger efficacy as likely reasons for a lack of resistance detected in greenhouse studies.

2.5.1 Gene targets

The gene targets we selected were based on results in other fungal systems; mutant studies in *S. musiva* were lacking. The decision to target *CYP51* and *DCL* was strongly influenced by the reported success of HIGS and SIGs against these gene targets in *Fusarium graminearum* (Koch et al., 2013) and *Botrytis cinerea* (Wang et al., 2016a). Whether *CYP51* and *DCL1,2* are essential in *S. musiva* remains unclear. Our initial efforts to screen dsRNAs (Figure S2.5) resulted in a lack of detectable growth reduction—which could mean either inefficient uptake, uptake but insufficient silencing, or uptake and silencing but a lack of growth phenotype. The sensitivity of *S. musiva* to demethylation-inhibitor fungicide (Figure S2.4) suggests that inhibition of *CYP51* could be an effective strategy if sufficient RNAi silencing could be achieved. Despite many published HIGS studies showing efficacy against fungal pathogens (Koch and Wassenegger, 2021a), relatively few target *CYP51*. Aside from *Fusarium* species (e.g., *graminearum* (Koch et al., 2013), *culmorum* (Koch et al., 2018), and *oxysporum* (Dou et al., 2020)), *CYP51* targeting has only been reported to be effective in one other species: *Magnaporthe oryzae* (Wang and Dean, 2022). Thus, further study is needed to better understand *CYP51* function in *S. musiva*, and thus to establish if this could be an effective gene target for HIGS. Thus, further study is needed to better understand *CYP51* function in *S. musiva*, and thus to establish if this could be an effective gene target for HIGS.
CYP51 copy number is variable among fungi and well known to positively correlate with azole fungicide resistance. To be an effective gene target for HIGS and SIGS, all functionally redundant copies need to be adequately silenced. For this reason, further identification of effective gene targets for HIGS and SIGS could focus on single-copy genes to simplify construct design. These could, for example, include key metabolic ("housekeeping") genes. Selection of genes that are moderately expressed (i.e., not too low or highly expressed) would also aid in assaying respective RNAi triggers in vitro with RT-qPCR (McLoughlin et al., 2018; Mosen, 2022).

Targeting components of the RNAi pathway (e.g., DCL), when the goal is to use that pathway against the fungus, appears counter-intuitive; however, doing so is reported to affect growth and or pathogenicity in several fungi. Our evidence suggests S. musiva DCL1 is not essential for conidial germination or mycelial growth in vitro. Our Sm-DCL-dsRNA suppressed DCL1 expression (Figure 2.1); however, the same treatment had no effect on culture density (Figure S2.5). It's possible that the degree of dc1 silencing was insufficient to observe a growth phenotype. Conversely, if DCL1 was sufficiently silenced, it's possible that any effect of silencing DCL1 could have been masked by a functionally redundant DCL2 that was not silenced by the RNAi trigger, despite being designed to target both copies.

Reduced vegetative growth in vitro due to knock-down or knock-out of fungal DCL genes appears to be relatively uncommon in fungi. Kettles et al. (2019) found no difference in growth rates of Zymoseptoria tritici mutants lacking core RNAi genes. Additional DCL mutant studies in Valsa Mali and Penicillium italicum also showed comparable in vitro growth rates to WT when cultured on rich media (Feng et al., 2017; Yin et al., 2020). Fusarium graminearum DCL mutants were reported to show normal vegetative development in axenic cultures in at least two studies (Son et al., 2017; Gaffar et al., 2019).

Growth phenotypes of DCL mutants in Botrytis cinerea, however, were less clear. Islam et al. (2020) found that dsRNA delivered in minicells to in vitro culture reduced growth,
but only when targeting both DCL1 and DCL2. Wang et al. (2016) claim attenuated growth of Botrytis cinerea DCL1 and DCL2 single and double mutants on malt extract agar; however, the evidence they present (image of unreplicated plates) lacked quantification or any measure of variation within groups (supporting figure 1a). Qin et al. (2023) generated their own B. cinerea DCL mutants and found no growth or morphology abnormalities relative to WT. He et al. (2023a) refute other conclusions of the Qin et al. (2023) study; however, they fail to address the contradictory B. cinerea DCL mutant growth phenotypes. One example of reduced in vitro growth comes from a Colletotrichum gloeosporioides double DCL mutant that showed attenuated growth compared to DCL single mutants and WT on both complete and minimal media (Wang et al., 2018b).

Regardless of whether DCL affects fungal growth, several studies suggest the better reason to target DCL is because it's involved in the biogenesis of siRNA effectors used in ckrNAi (Göhre and Weiberg, 2023). SIGS and or HIGS against fungal DCL genes is reported to reduce pathogenicity of Botrytis cinerea (Wang et al., 2016a), Verticillium dahliae (Wang et al., 2016a), Fusarium graminearum (Werner et al., 2020), and Penicillium italicum (Yin et al., 2020). Additionally, reduced pathogenicity of mutant strains missing one or both DCL genes has been demonstrated in Botrytis cinerea (Weiberg et al., 2013), Valsa mali (Feng et al., 2017), Colletotrichum gloeosporioides (Wang et al., 2018b), and Penicillium italicum (Yin et al., 2020).

The extent of siRNA effector use among fungi as a virulence strategy is unclear (Mahanty et al., 2023a). One fungus that does not appear to use siRNA effectors is Zymoseptoria tritici, a close relative to S. musiva that shares a hemibiotrophic lifestyle (Kettles et al., 2019; Ma et al., 2020). Kettles et al. (2019) demonstrate that Z. tritici missing core RNAi genes were just as virulent on wheat as WT and conclude that RNAi plays a minimal role during infection. Whether or not S. musiva DCL is involved in siRNA effector biogenesis clearly requires further study. Generation of core RNAi gene knockouts in S. musiva, along with virulence assays, would be helpful in establishing the role of RNAi during infection. Specifically, pathogenicity screening of S. musiva DCL
mutants paired with RNA sequencing could help determine if siRNA effectors used in ckRNAi contribute to virulence.

2.5.2 RNAi triggers: design and screening
Our two RNAi constructs targeted a total of five *S. musiva* genes, yet only one transcript was silenced *in vitro* (Figure 2.1). This result suggests at least some dsRNA was being taken up by *S. musiva* but that dsRNAs targeting the other four transcripts were not generating efficient siRNAs. Variation in silencing efficiency among siRNAs targeting the same mRNA is common (Holen, 2002). One reason why only some siRNA are effective can be explained by mRNA secondary structure that limits access of the RNAi-induced silencing complex (RISC) to the target site (Shao *et al*., 2007). The design tool we used to select construct sequences does use mRNA structural predictions to determine which regions are most accessible to RISC (Lorenz *et al*., 2011; Lück *et al*., 2019). However, *in silico* structure predictions remain imperfect as they lack important information on possible mRNA modifications that are known to influence secondary structure (Tanzer *et al*., 2019). A study by Werner *et al.* (2020) also tested dsRNAs designed with the same software used here and found a mix of ineffective and potent silencing efficiencies among constructs targeting *Fusarium graminearum* genes *in vitro*. They found that tool-designed dsRNA constructs kept short (173-193 bp) to minimize non-target silencing tended to be less effective than manually selected longer sequences (658-912 bp) at triggering silencing in *Fusarium graminearum*. Longer randomly selected dsRNAs tended to provide greater numbers of efficient siRNA that led to stronger silencing. The same group found that dsRNA lengths up to 1500 bp were negatively correlated with SIGS-mediated disease resistance, which suggests dsRNA length eventually hinders fungal and or plant uptake (Höfle *et al*., 2020). The length of our dsRNAs were between 645 and 891 bp, and each target sequence within these stacked constructs were between 251 and 315 bp (Figure S2.3). Further study of single gene *S. musiva* constructs with longer sequences could determine if alternate target sites would be more efficient at triggering RNAi.

Our *in vitro* assays demonstrated the challenges with screening dsRNA triggers in pure culture. We found that the microwell axenic culture growth inhibition assay described by
Koch et al. (2013) to be problematic with *S. musiva* due to secretion of RNase (Figure S2.6). Two SIGS studies suggest dsRNA degradation in *Botrytis cinerea* cultures might also be occurring as more efficient silencing was observed when dsRNA was applied every 12 hours (Song et al., 2018; Nerva et al., 2020). Conversely, single dsRNA additions at relatively low concentrations (0.5 ng/µl) have been shown to knock-down target gene expression for 96 hours in *Sclerotinia sclerotiorum* (McLoughlin et al., 2018). The dsRNA culture concentration used by Koch et al. (2013) to measure *in vitro* knock-down at 72 hours was nearly 150 times greater than the dose used on *Sclerotinia sclerotiorum* by McLoughlin et al. (2018). In the case of *S. musiva*, the dose used in our growth inhibition assay was 20 times the dose used by McLoughlin et al. (2018) and disappeared in culture supernatant after 24 hours, while the same dose increased by 10-fold remained for 48 hours in *S. musiva* culture. The *S. musiva* culture filtrate that also quickly degraded dsRNA came from potato dextrose broth culture that was not treated with dsRNA (Figure S2.6b). This result suggests *S. musiva* doesn’t require dsRNA to induce secretion of dsRNA-degrading nuclease under the culture conditions, although induced secretion triggered by trace amounts of potato dsRNA remaining in the culture medium can’t be ruled out.

*Sphaerulina musiva* and the fungi mentioned above all employ either full or delayed necrotrophic host colonization and therefore secretion of catabolic enzymes would not be surprising for any of these fungi. With the range of dsRNA doses reported for *in vitro* RNAi, it's unclear the degree to which higher doses reflect a need to overwhelm secreted nuclease and or whether the range of doses reflect differences in the efficiency of dsRNA uptake and silencing (Qiao et al., 2021). Further *in vitro* screening of RNAi triggers against *S. musiva* might be improved with the use of RNase inhibitors if cytotoxic side effects can be avoided. For example, we showed that proteinase K could protect dsRNA from degradation by culture filtrate. Should *S. musiva* tolerate proteinase K in culture, it might be used to protect dsRNA, and thus improve *in vitro* uptake and silencing. We found EDTA, a chelating agent used to inhibit RNase and present in dsRNA annealing buffer preparations, prevented germination in potato dextrose broth at 1 mM (unpublished). Various nano-carriers have been shown to offer protection from nuclease,
and many of these also aid uptake into plants to elicit RNAi that might also aid fungal transfection (Ray et al., 2022). These include "BioClay" (Mitter et al., 2017), carbon-dots (Schwartz et al., 2020), and cell penetrating peptides (Numata et al., 2014) among several others. Further study is needed to see if any of these formulation strategies could aid dsRNA stability and uptake in S. musiva culture.

2.5.3 Host transgene expression:resistance relationships?
Although no resistance was detected in greenhouse trials (Figures 2.3, 2.6), transgene expression profiling provided assurance that the dual inverted promoter constructs were expressed and that enough high expression events were challenged to adequately test HIGS against our gene targets (Figures 2.4, 2.5). Correlations of HIGS transgene expression and disease severity served as another line of evidence to suggest whether HIGS could explain variation in the disease response (Figure 2.7). We hypothesized that the highest expression lines would be capable of delivering the highest dose of dsRNA, and therefore would have the highest potential for resistance. Expression of HIGS transgenes did have a significant relationship with greenhouse resistance, but only among Sm-DCL-expressing transgenic events, and not among Sm-CYP51-expressing events (Figure 2.7). The significant correlation suggests that some level of HIGS might have been active in higher Sm-DCL-expressing events. These events targeted S. musiva DCL1 and DCL2, the former of which showed significant knock-down in vitro by the same dsRNA trigger (Figure 2.1). Nonetheless, even the high expression lines did not have detectably improved resistance in our greenhouse screens. It’s possible that our dsRNA constructs were inefficient or that a much higher number of lines need to be screened to find very high expressors. We conclude that HIGS was not contributing to variation in disease severity that was strong enough to detect under our experimental conditions. This may be a result of inadequate dsRNA expression, limited fungal uptake, uptake but insufficient silencing, greenhouse conditions being much more conducive to disease than might occur in field-grown trees, or silencing but the gene targets were not essential to growth and pathogenicity.

Dual inverted promoter constructs are generally preferred over more traditional hairpin designs because of the relative ease of cloning (Schmidt 2012), and as desired gave a
wide range of transgene expression among the HIGS events (Figure S.2.2a). This style of construct has been effective in inducing HIGS in *Arabidopsis* and barley (Koch 2013) and has also been shown to be as effective as hairpin designs in knocking down transgenes (*GUS*) in *Nicotiana tabacum*, or endogenous genes in hybrid aspen (Schmidt 2012). The variable transgene expression (Figure 2.4, 2.5) is assumed to reflect position effects, copy number, and or transgene silencing resulting from repetitive elements (Birch, 1997; Kooter *et al*., 1999, Kohli *et al*., 2003). Determining tradeoffs in silencing potential would be useful for the continued development of RNAi-mediated resistance in *Populus*. Bisulfite-seq of transgene insertions could help to explain variation in transgene expression, with a negative correlation of promoter DNA methylation and expression expected if epigenetic silencing was important. Furthermore, contrasts to events transformed with comparable hairpin constructs would be useful in evaluating whether opposing dual-inverted RNAi vectors are especially prone to transcriptional gene silencing, an observation supported by the very high number of lines with nearly undetectable expression compared to that from the selection marker genes delivered in the same T-DNAs (Figs. 2.4 and 2.5).

The methods we used to estimate dsRNA expression among HIGS transgenic lines may not have been fully reliable measures of potential disease resistance. First, HIGS transgene expression levels represent those of ramets in *in vitro* culture and are assumed to be the same among clones acclimated to greenhouse conditions. A study of transgene expression in hybrid poplar (*Populus tremula* x *P. alba*) found that expression increased from *in vitro* culture to greenhouse conditions in some lines while it decreased in others (Hawkins *et al*., 2003). Instability of transgene expression has also been described for *ROLC* transgenic aspen and aspen hybrids (*P. tremula* L. x *P. tremuloides* Michx.) after transfer from *in vitro* conditions to greenhouse (Kumar and Fladung, 2001). Second, our methods didn't measure dsRNA directly. This limitation was the reason we used the term "HIGS transgene expression" rather than “dsRNA expression” to describe expression profiling of HIGS events. Priming could occur on either sense or antisense dsRNA precursors. The assumption is that these were present in equimolar quantities and annealing to form dsRNA. Shorter dsRNAs including siRNAs derived from host-DCL
processing of full-length dsRNA precursors could not be amplified with this approach. The priming strategy targeted 119 and 156 bp sections of the long sense and/or antisense dsRNA precursors (p9U10-RNAi-Sm-CYP51 and p9U10-RNAi-Sm-DCL respectively). Thus, the relative expression of HIGS transgenes captured by our method is assumed to represent the cellular pool of unprocessed dsRNA, which should be a fraction of the actual dsRNA expression level that includes diced dsRNA.

Both long dsRNA and siRNA are known to contribute to HIGS, though it's unclear which contributes more to silencing (Karimi and Innes, 2022). It's possible that lines with low measured dsRNA expression could have had higher siRNA expression if host DCL processing was more efficient in these lines. If this were true and siRNA contributed more to HIGS in this pathosystem, then we might expect more resistance in our "low expression" lines; however, the significant negative correlation between Sm-DCL dsRNA expression and greenhouse disease severity is inconsistent with this hypothesis, though both specific low and high expressors could cause strong resistance, confounding a simple linear model.

Alternate methods to measure dsRNA expression could be useful. Northern blotting and related dot- and slot-blotting that are more readily quantified remain useful techniques that could be used to validate both dsRNA and siRNA expression. Stem-loop RT-qPCR is one method capable of amplifying siRNA (Varkonyi-Gasic et al., 2007). However, priming for this method is specific to individual siRNA and therefore isn't suited to amplification of the many possible siRNA that can be generated from a several hundred base pair long dsRNA. RNA-seq is another option for profiling dsRNA expression across events, although costly, and is not necessarily needed if phenotypic resistance is available to screen events for functional expression. One study demonstrated that small RNA-seq (sRNA-seq) could be used on dsRNA treated in vitro culture to sequence siRNA and map them back to the long dsRNA precursor to suggest preferential host DCL processing and illuminate siRNA "hot spots" within an RNAi construct (Koch et al., 2016). If "hot spots" were consistent across events, then stem-loop RT-qPCR could be used to find these siRNAs and might be a better proxy for dsRNA expression than the methods used here.
However, Koch et al. (2016) caution that "hot spots" might also reflect priming and sequencing bias.

2.5.4 Fungal dsRNA uptake
Several authors have suggested dsRNA uptake as the most important success factor in either HIGS or SIGS approaches against fungi (Koch and Wassenegger, 2021a; Qiao et al., 2021; Šečić and Kogel, 2021). The only evidence we found for uptake was repeated knock-down of one gene target in vitro (Figure 2.1). However, direct observations of uptake using fluorescently labeled dsRNA and live imaging suggest efficiency is low, and that in vitro knock-down may have only been observed because of the high dsRNA dose needed to overwhelm secreted RNase activity in culture (Figure S.2.6). In contrast to fungi such as B. cinerea that are known to readily take up environmental dsRNA (Wang et al., 2016a), uptake by S. musiva appears relatively inefficient (Figure 2.2.). Using similar methods, Qiao et al. (2021) found a range of uptake efficiencies amongst seven fungi. They also found that strong environmental dsRNA uptake predicted efficient target gene silencing and effective SIGS control of fungal disease caused by Botrytis cinerea, Sclerotinia sclerotiorum, Rhizoctonia solani, Aspergillus niger and Verticillium dahliae. Conversely, they also found that weak uptake predicted inefficient gene silencing and SIGS-mediated disease suppression in Colletotrichum gloeosporioides.

Another example of inefficient environmental dsRNA uptake associated with unsuccessful RNAi-mediated disease suppression comes from Kettles et al. (2019), who report a lack of detectable uptake of fluorescently labeled dsRNA in Zymoseptoria tritici, as well as no resistance mediated by virus-induced gene silencing (VIGS) against essential genes. They conclude Z. tritici has low potential for RNAi-mediated control. Given the relatedness of Z. tritici to S. musiva, their similar hemibiotrophic infection modes, and comparable poor dsRNA uptake, these fungi may share mechanisms that reduce uptake of exogenous dsRNA. Aside from these examples in C. gloeosporioides, Z. tritici, and S. musiva, we are unaware of any other reports of inefficient dsRNA uptake in fungi. Uptake efficiencies for the vast majority of phytopathogenic fungi remain to be described (Wytinck et al., 2020a). Although much research remains to be done, the publication of many successful HIGS applications may reflect the well-known, and
extensively documented publication bias towards significant findings (Chong et al., 2023).

The cellular mechanisms by which fungi take up dsRNA are still being discovered and debated (Wytinck et al., 2020a; Koch and Wassenegger, 2021a; Šečić and Kogel, 2021; Karimi and Innes, 2022; He et al., 2023). Mechanisms for uptake from HIGS plants may be different than those used for uptake of environmental dsRNA. For example, if plant-produced dsRNAs exported to the extracellular space are bound by protein and or packaged in extracellular vesicles (EVs), then uptake through the fungal cell wall and membrane may work with different cell receptors and transporters than those used during the uptake of exogenous naked dsRNA—as in the case of uptake from a leaf surface or in vitro culture. Protection of dsRNA by bound protein or encapsulation in EVs might also protect it from nuclease degradation. It's therefore plausible that a fungus showing weak uptake of environmental dsRNA might still show sufficient dsRNA uptake from a HIGS plant to induce RNAi. This might be the case for Colletotrichum gloeosporioides where one study found inefficient uptake both in vitro and in planta on SIGS treated fruits (Qiao et al., 2019) while another study reports effective HIGS disease suppression in chili and tomato (Mahto et al., 2020). However, the gene targets were different in the two studies.

It's not clear from our greenhouse experiments whether S. musiva was taking up dsRNA from HIGS plants—as would strongly be suggested if we observed resistance. Our greenhouse screening methods had a lot of variability within replicated clones (CV2020 experiment = 43.7, CV2022 experiment = 62.4) and thus power to detect only very large effects, such as when we compared resistance to that in non-host species (Figure S2.7). Additionally, in our greenhouse studies, inoculum conditions and plant physiology were likely much different than what would be encountered in field settings, which may have overwhelmed small effect sizes. It's therefore possible that HIGS effects might have been detected under field conditions. Nevertheless, an absence of HIGS resistance does not rule out dsRNA uptake, as uptake with ineffective RNAi trigger sequences and or gene targets could also explain this result. Further study of target gene knock-down in infected
tissue, preferably both in vitro and in vivo, is needed to assess the extent of S. musiva uptake during host colonization.

2.5.5 Conclusion
This study represents the first known attempt to develop HIGS in a forest tree against a fungal pathogen. Our very limited ability to detect dsRNA uptake by S. musiva, and the lack of detectably resistant transgenic plants produced, suggest that HIGS is unlikely to be an effective means of disease control in this pathosystem.

2.6 References


3. Effects of *in vitro* culture, transformation, and RNA interference on foliar fungal microbiomes in poplar

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3.1 Abstract

Plant transformation is a common means for gene insertion and editing during scientific research and biotechnology; however, its effects on plant microbiomes are unknown. We used ITS metabarcoding to evaluate the effects of transformation including organogenesis, propagation, and field acclimation—on the foliar fungal microbiome in poplar trees and at a field site over two growing seasons. Sterile, micropropagation-derived trees grown in a greenhouse were largely uncolonized after 50 days. Once established in the field, fungal communities were nearly as diverse as in well-established trees of the same genotype after one season of growth. We did not detect effects of micropropagation, or the use of antibiotic selection and organogenesis, on subsequent foliar fungal communities. The expression of gene silencing-inducing constructs directed against the Septoria canker fungus also had no detectable effect on non-target communities. Our results suggest that micropropagation, genetic transformation and associated antibiotic selection and organogenesis, and the expression of antifungal RNAi, had minimal, if any, impacts on subsequent foliar microbiomes of field-grown *Populus*.

3.2 Introduction

Poplars are hosts to taxonomically and functionally diverse microbiomes (Hacquard and Schadt 2015) that have important impacts on plant fitness and health (Trivedi *et al.*, 2020). For instance, commonly occurring fungal leaf endophytes in poplar can alter the severity of disease caused by both biotrophic and hemibiotrophic foliar pathogens (Busby *et al.*, 2013, 2016). In addition to species interactions, microbiome composition is also affected by genotypic variation within host plants. For example, the genes involved in cuticle composition, chitin/flagellin detection, plant cell wall modification, and signal transduction have been shown to impact microbiome composition, and many of these genes are also involved in pathogen defense (Bressan *et al.*, 2009; Balint-Kurti *et al.*, 2010; Bodenhausen *et al.*, 2014; Horton *et al.*, 2014; Ritpitakphong *et al.*, 2016). It is therefore reasonable to expect that alterations to plant disease resistance through transgenic or gene editing methods could have impacts on microbiome structure and the disease resistance they influence.
The methods employed for genetic transformation and gene editing typically cause significant physiological disruptions to plants. The source material for genetic transformation is typically derived from surface-sterilized plants micropropagated in in vitro culture (Ma et al., 2004); their microbiomes are absent or greatly reduced in complexity (Orlikowska et al., 2017). Repeated clonal propagation, particularly when it involves de novo callogenesis, followed by organogenesis or embryogenesis in the presence of plant growth hormones such as strong auxins, is well-known to generate aberrant phenotypic changes often referred to as somaclonal variation. Gene insertion also causes diverse mutations near to the insertion site that are known to include indels, duplications, and other rearrangements that can lead to transgene silencing and affect expression of nearby genes (Potrykus 1991, Birch 1997). Antibiotics are also typically used to enrich for modified cells, which can directly affect microorganism populations and induce random genome-wide mutations and epigenetic modifications (Bardini et al., 2003). Thus, in addition to the new properties imparted to plants by gene insertion or editing, the transformation process may affect microbiomes in the short and long term.

Host-induced gene silencing (HIGS) is an emerging biotechnology that has been used to develop heritable resistance to specific fungal pathogens in diverse pathosystems (Nagle et al., 2018). In contrast to broad spectrum fungicides, HIGS can be highly specific to the target organism. HIGS relies on constitutive host-expression of transgenic double-stranded RNAs (dsRNAs) complementary to essential transcripts in the fungal pathogen. The target pathogen must subsequently take up dsRNA from host tissue during infection through natural mechanisms of nucleic acid exchange (Cai et al. 2018). Once taken up, dsRNAs trigger target gene silencing via conserved fungal RNA interference (RNAi) pathways, thereby limiting growth and virulence. Silencing of pathogen genes requires sequence complementarity between dsRNA transgenes and the target transcript. Silencing can still be achieved with mismatches, although specific tolerances in fungi are not as well studied as those in mammals, plants, and invertebrates (Machado et al., 2018; Chen et al., 2021).
To avoid off-target silencing in the host and non-target silencing in other species when using HIGS, dsRNAs are typically designed to target the non-conserved regions of essential housekeeping or virulence genes where DNA sequence conservation between species drifts rapidly relative to conserved domains. Most research in this field assumes that the sequence-specific mechanism of RNAi combined with careful gene target and sequence selection should mean that HIGS will be highly specific to the targeted organism. However, this has not been tested.

Moreover generally, few studies have tested effects of transformation on plant microbiomes. Most have focused on the rhizosphere rather than the phyllosphere (Stefani and Hamelin., 2010; Wei et al., 2006; Vauramo et al., 2006; Seppänen et al., 2007; Kremer, 2020); though, both are host to diverse communities with the potential for perturbations caused by plant transformation or HIGS. For example, transgenic American chestnut expressing oxalate oxidase (OxO) had no effect on colonization by ectomycorrhizal fungi (Dulmer et al., 2014; D'Amico et al., 2015; Newhouse et al., 2018). The authors suggested that the absence of effects could be attributed to the widespread distribution of OxO enzymes in plants and the lack of toxic effects of these enzymes on ectomycorrhizal fungi. Similarly, Stefani et al. (2009) found no effects of selectable marker expression (NPTII) or reporter gene expression (GUS) on ectomycorrhizal fungal communities of poplar trees in an eight-year-old plantation. Conversely, studies in GE crops and trees suggest that transgene exudates (e.g., insecticidal Bt toxins) can shape rhizosphere community structure (Colombo et al., 2020), though effects can be transient (Wu et al., 2020) and depend on the transgene and host (Stefani and Hamelin, 2010). While mutations and phenotypes arising from somaclonal variation and plant transformation are well known (Bhojwani and Dantu, 2013), we are not aware of studies that have looked at perturbations to the microbiome from transformation under field conditions, nor as a result of expression of dsRNAs intended to cause HIGS against an important disease of poplars.

Using ITS metabarcoding, we tested the overarching hypothesis that transgenic methods of imparting HIGS would affect foliar fungal endophyte communities of poplars.
Specifically, we tested three hypotheses: 1) a history of micropropagation would affect foliar fungal community composition and richness; 2) plant transformation methods, including a history of organogenesis and exposure to antibiotic selection, would affect fungal community composition and richness; and 3) non-target effects of HIGS against a specific fungal pathogen would be limited to foliar fungal endophytes with conserved target sequences. To our knowledge, this study is the first of its kind to evaluate the effects of transformation on foliar fungi, and to evaluate the specificity of HIGS against fungi under field conditions.

3.3 Methods

3.3.1 Study population
We evaluated foliar fungi on field-grown transgenic and non-transgenic plants that were part of an effort to develop HIGS-mediated resistance to *Sphaerulina musiva* in *Populus trichocarpa* (Chapter 2). All genotypes were of the *S. musiva* susceptible background SLMB 28-1 (T-61; Table 3.1). HIGS against *S. musiva* was designed to target homologs of gene targets found effective in published HIGS studies against *Fusarium graminearum* and *Botrytis cinerea* (Koch *et al.*, 2013; Wang *et al.*, 2016). RNAi constructs target either cytochrome p450 lanosterol C-α-demethylase involved in ergosterol biosynthesis (*Sm-CYP51*), dicer-like genes (*Sm-DCL*), or as a negative HIGS control, the reporter gene beta-glucuronidase (*GUS*) (Figure S2.2). All gene-targeting cassettes were synthesized and cloned into vectors p9U10-RNAi and p6U10-RNAi by DNA Cloning Service (Hamburg, Germany). Transformations were made using standard *Agrobacterium* methods (Ma *et al.*, 2004; Song *et al.*, 2006; Li *et al.*, 2017). Shoots forming under geneticin (kanamycin) selection (p9U10-RNAi-Sm-CYP51 and p9U10-RNAi-GUS events) and (or) hygromycin selection (p6U10-RNAi-Sm-DCL events) were PCR confirmed for the full-length dsRNA transgenes. A total of 77 HIGS events were generated including 37 p9U10-RNAi-Sm-CYP51 events, 27 p6U10-RNAi-Sm-DCL events, nine events co-transformed with both p9U10-RNAi-Sm-CYP51 and p6U10-RNAi-Sm-DCL, and four p9U10-RNAi-GUS control events. Expression of HIGS
transgenes was validated for all independent events using RT-qPCR as discussed in chapter 2 (Figures 2.4 and 2.5).

Several control types were included to test for effects of plant transformation methods and micropropagation. All controls were generated at the same time as the HIGS lines. Three events were included that only express the selectable marker hygromycin B phosphotransferase (*HPT*). Four non-transgenic “escapes” (NTE) were included that went through the entire transformation process. Each NTE came from an independent regenerated shoot with ineffective antibiotic selection. Antibiotic selection was imposed as follows: We used 5 mg/L kanamycin in callus induction media for 21 days in the dark, 10 mg/L in shoot induction media for three to four months in the light, 10 mg/L in the shoot elongation media in light for two months, and 25 mg/L for rooting. In escapes, the absence of both dsRNA and selectable marker transgenes and their transcripts was verified with PCR and RT-qPCR respectively. Finally, there were three *wild type* (*WT*) controls that included *WT* with a three-year history of micropropagation only (*WT*-in *vitro*) (it was also the explant source for HIGS transgenics), *WT* propagated in a greenhouse from dormant cuttings collected from a clone bank (*WT*-ex *vitro*), and *WT* sampled directly from an established clone bank neighboring the HIGS common garden (*WT*-clone bank).

### 3.3.2 Poplar culture

Prior to the field planting, ramets were multiplied in tissue culture on woody plant media with rooting hormone (WPM-RT). Micropropagated ramets were acclimated to soil (SunGro Sunshine #4) in 2¼ x 3¼ inch pots (Anderson #1683) placed in sealed 1-gallon plastic bags and grown with an 18-hour photoperiod for three weeks in a headhouse. Ramets were acclimated by first opening plastic bags for one week, then pots were transferred into translucent plastic tubs with slightly opened lids in a greenhouse for two additional weeks while lids were gradually removed over the first week. Acclimated greenhouse plants were potted in 2.5 x 10-inch Deepot cells. Plants were fertilized with slow-release granules mixed into the soil at 7.2 g/l of soil (Harrell's 16-6-12 Polyon Nursery). Ramets were kept singled under an 18-hour photoperiod until reaching an
average height of approximately 30 cm. Trees were then acclimated to outdoor conditions in a lathouse for three weeks prior to field planting.

3.3.3 Common garden
HIGS transgenic lines and controls were established at a field site in Corvallis, OR on June 4, 2021. Nutrient poor soils were amended during planting. An auger was used to dig holes to a standardized depth of 0.5 m (hole volume approximately 15 liters). Each planting received 2 liters of SunGro Sunshine #4, 12 liters of compost (Magik Grow Soil, The Bark Place, Corvallis, OR), and then a final top dressing of site soil displaced by the auger. Plantings were randomized into a single block with 1.5 m spacing between rows and columns (21 x 25). Trees received overhead irrigation by impact sprinklers at least every other day and up to once a day as needed for one hour in the morning. A grassy ground cover was cut periodically with a mower.

Genotypes were replicated at the level of line, and lines (i.e., transgenic insertion events) were replicated at the level of ramet. To control for variable fertility across the field plot, we used a completely randomized block design. To further control for within block variation of fertility, we included a covariate seasonal increase in height as a proxy for fertility.

3.3.4 Sample collection and DNA extraction
Ramets were sampled in tissue culture, in the greenhouse prior to field planting, and again in the field over two seasons. To determine whether our WT source material for explants carried background fungal communities, we sampled whole leaves from the in vitro culture used to propagate field ramets. Sampling was focused on control genotypes used in the field study to avoid confounding effects of dsRNA expression (Table 3.1). In each sample, three leaves were collected from individual ramets from the top, middle and lower sections of the stem. In vitro leaf samples were not washed to remove surface microbes as below for greenhouse and field samples due to the delicate nature of the tissues. To assess colonization of leaves in the greenhouse, leaves were sampled from control ramets after 50 days of greenhouse growth and processed as noted below for field samples. To minimize foliar fungal growth during greenhouse propagation, plants were
watered by hand wand with water directed at the soil. Field-grown leaves were sampled after one season of growth on September 29th, 2021, and again the following year on September 23rd, 2022. These dates occurred prior to obvious signs of leaf senescence. In addition to sampling at the field site described above, three WT clones with the same SLMB-28-1 background were also sampled on the same days from an established clone bank adjacent to this field site to provide a relative measure of colonization.

The sample unit was one tree, represented by the aggregation of twelve leaf discs. From each tree, three leaves were sampled at the 5th, 6th, and 7th position down from the meristem. Within each leaf, two punches were made haphazardly on each side of the midvein using an office hole punch (6 mm diameter) rinsed and sanitized with 70% ethanol in between samples. Samples were stored on ice until transport to the lab, and then at 4°C for less than 24 hours before further processing. To remove debris and surface microbes, leaf discs were washed with 0.1% v/v Triton X-100 under gentle agitation (500 RPM) in a GenoGrinder 2010 (Spex SamplePrep, Metuchen, NJ, USA). Washing was followed by two rinses with molecular grade water under gentle agitation before storing at -80 °C. DNA was extracted with 96 Well SYNERGY™ Plant DNA Extraction Kit (OPS Diagnostics SYNP 02-96-03, Bridgewater, NJ, USA) using manufacturer’s instructions. Samples were homogenized in a GenoGrinder 2010 for 15 min at 1,500 RPM. Three empty wells per 96-well plate were left as negative extraction controls.

3.3.5 Library prep and sequencing
Fungal amplicon libraries were prepared for Illumina MiSeq using a two stage PCR approach. The first PCR reaction targeted the ITS2 region using forward primer ITS3_kyo1 (Toju et al. 2012) and reverse primer ITS4 (White et al. 1990). Stage one primers included Illumina priming sites and 3-6 bp, degenerate, length-heterogeneity spacers (Lundberg et al. 2013) intended to increase library complexity. Stage one PCR reactions were 25 µl and included Bioline MiFi 2x Master Mix, 0.5 µM of each primer, 5 µl of template, and 1 µM peptide nucleotide acid oligo blocker (PNA clamp) to inhibit co-amplification of P. trichocarpa 5.8S nrRNA genes (Cregger et al. 2018). Reaction conditions for stage one were: 95 °C for 3 min., 28 cycles of 95 °C for 30 sec., 78 °C for
5 sec., 50 °C for 30 sec., and 72 °C for 30 sec., followed by a final elongation cycle at 72 °C for 5 min.

Stage two PCR served to dual index samples with 8-mer multiplexing barcodes (Hamady et al. 2008) and add P5 and P7 Illumina adapters by priming off the Illumina priming sites added in stage one. Stage two PCR reactions were 25 µl and included Bioline MiFi 2x Master Mix, 1 µM of each primer, and 1 µl of stage one PCR product. Reaction conditions for stage two were: 95 °C for 1 min., 8 cycles of 95 °C for 20 sec., 55 °C for 20 sec., and 72 °C for 30 sec., followed by a final elongation cycle at 72 °C for 5 min.

Agarose gel electrophoresis was used to evaluate size and quality of both PCR reactions. Stage two PCR amplicons were cleaned and adjusted to equal concentrations prior to sample pooling using the Just-a-Plate™ 96-well PCR cleanup and normalization kit (Charm Biotech, San Diego, CA, USA). Libraries were sent to Oregon State University Center for Quantitative Life Sciences, for 250-bp paired-end sequencing on their Illumina MiSeq platform.

3.3.6 Sequence processing and filtering

Primers were removed using Cutadapt v3.5 (Martin 2011) and SeqPurge v2021_12 (Strum et al. 2016). Sequences were further processed and analyzed in R v 4.3.1 (R Core Team 2023). A sequence table was generated using a standard DADA2 (Callahan et al., 2016) workflow to trim low-quality base calls, denoise, merge, and remove chimeras. Fungal ITS sequences >50 bp were extracted with ITSx (Bengtsson-Palme et al. 2013) and taxonomy assigned with DADA2 using the UNITE fungal database v8.2 (Nilsson et al., 2018). Further taxonomic assignments were manually curated using BLASTn queries in GenBank. Spurious amplicon sequence variants (ASVs) matching P. trichocarpa genome v4.1 were removed using bowtie2 v2.4.5 (Langmead and Salzberg 2012). Operational taxonomic units (OTUs) were generated by clustering ASVs at 97% similarity with single linkage method in the R package DECIPHER v2.22.0 (Wright 2016). Regardless of species prevalence, all OTUs were retained to avoid losing information of possible relevance to the question of non-target effects. Samples with low sequencing depth (<500 reads) were removed except in the analysis of tissue culture and greenhouse samples, in which all samples were analyzed regardless of depth. Read counts
were relativized by sample totals to normalize variable sequencing depth. Taxonomy, sequence counts, and sample metadata were combined into a single object in the R package phyloseq for downstream analysis (McMurdie & Holmes, 2013).

3.3.7 Data analysis
Year one and year two field data were analyzed separately. Taxonomic composition and OTU richness were estimated and plotted using phyloseq. To compare WT OTU richness in consecutive years in the HIGS common garden, we rarefied samples to 2,500 reads and tested for differences with two sample t-tests. The same methods were used to test for differences in OTU richness between WT genotypes in the HIGS common garden and WT in the established clone bank.

To visualize differences in fungal community composition among field genotypes, sample units were ordinated in OTU space using non-metric multidimensional scaling (NMDS) of Bray-Curtis dissimilarities using the R package vegan (Oksanen et al., 2007). Bray-Curtis distance was calculated from OTU counts relativized by sample totals to normalize variable sequencing depth. Ordination plots with color overlays for genotype were produced with phyloseq.

Group comparisons that addressed specific hypotheses are outlined as follows. To test the hypothesis that a history of micropropagation has no effect on foliar fungal community composition, we compared communities of WT-in vitro to WT-ex vitro. To test the hypothesis that plant transformation methods, including a history of organogenesis and exposure to antibiotic selection have no effect on fungal community composition, we contrasted non-transgenic escapes (NTE) with WT samples that were only micropropagated (WT-in vitro). To test the hypothesis that HIGS targeting S. musiva genes will have no effect on non-target fungal communities, we compared S. musiva targeting genotypes (Sm-CYP51, Sm-DCL, Sm-CYP51+Sm-DCL) with control genotypes not targeting S. musiva genes (GUS, HPT, NTE, WT-in vitro, WT-ex vitro). Comparisons of the same groups were used to test the hypothesis that non-target effects of HIGS would be limited to closely related species. When testing for effects of HIGS, we also analyzed a reduced data set that only included seven p6U10-RNAi-Sm-DCL events with high
relative expression of the HIGS transgene compared to other events (Figure 2.5). These high expression events were contrasted with transgenic controls expressing non-specific dsRNA (four p9U10-RNAi-GUS events).

To test for differences in community composition in the above contrasts, we used permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) of Bray-Curtis dissimilarities. We used Primer v7/PERMANOVA+ (Anderson et al., 2008) to run PERMANOVA tests with partial sum of squares and 9,999 permutations. PERMANOVA models partitioned variance by fixed effect of block (n = 4), covariate seasonal increase in height, and fixed effect of treatment. When modeling effects of HIGS, effects of independent transgenic lines were modeled as random effects nested within fixed effects of genotype (RNAi construct). Given that PERMANOVA results are sensitive to group differences in multivariate dispersion, we used the PERMDISP test (Anderson 2006) in Primer/PERMANOVA+ to evaluate the hypothesis of multivariate homogeneity of group dispersions. In addition, to evaluating differences in community composition for each contrast, we also tested for differences in OTU richness using the methods described above for WTs in consecutive sampling years.

To test our hypothesis of non-target effects being limited to close relatives of *S. musiva*, we used MUSCLE (Edgar, 2004) to align *S. musiva* target gene transcripts with available homologous transcripts of some of its known antagonists (P. Busby, Oregon State University, unpublished). Alignments spanned the areas targeted by our RNAi constructs and were manually inspected for regions of contiguous sequence conservation that might result in non-target silencing.

To look for differential abundance of individual taxa between *S. musiva*-targeting genotypes and non-*S. musiva* targeting controls, we generated effect sizes and confidence intervals (95% C.I.) using ANOVA-like differential expression analysis (ALDEEx) methods within the R package ALDEEx2 (Fernandez et al., 2013). ALDEEx takes an OTU count matrix and performs a centered log-ratio (CLR) transformation to address the compositional nature of sequencing data. Technical variability across OTUs within each
sample is estimated using Monte-Carlo instances drawn from the Dirichlet distribution, and repeated sampling from this distribution gives a posterior probability distribution of the observed data (Fernandez et al., 2013). Genotype effects were estimated by computing the median standardized difference of CLR values between groups, after accounting for block effects. An OTU was deemed differentially abundant if the confidence interval (±two standard errors) did not encompass zero.

**Table 3.1 Summary of study population**

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<thead>
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<th>genotype</th>
<th>regeneration &amp; selection</th>
<th>transgenic</th>
<th>HIGS</th>
<th>S. musiva targeting</th>
<th>micro-propagated</th>
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<th>ramet (n)</th>
<th>total</th>
<th>line (n)</th>
<th>ramet (n)</th>
<th>total</th>
<th>line (n)</th>
<th>ramet (n)</th>
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### 3.4 Results

#### 3.4.1 Sequencing stats and gamma diversity

The first sequencing run, including *in vitro*, greenhouse, and year-one field samples yielded a total of 3.34x10^6 high-quality filtered and merged reads over 383 samples, and the second sequencing run, including only year-two field samples, yielded 5.13x10^6 high quality merged reads over 373 samples. After filtering host-amplification, short reads, non-fungal reads, and samples below the minimum depth (6 samples in year-1, 161 samples in year-2), the final ASV matrix was reduced to 1.76x10^6 total reads in year-1 (field sample median = 5,567) and 3.32x10^6 reads in year-2 (sample median = 13,307). In year-1, a total of 534 fungal ASVs were detected that clustered into 372 OTUs, and in year-2, a total of 808 fungal ASVs were detected that clustered into 548 OTUs. The large number of samples with under 500 reads in year-2 was attributed to variable yield and quality of DNA extracts.
3.4.2 Minimal fungal colonization detected in tissue culture and greenhouse samples

We detected minimal colonization in our in vitro plants. Of the forty in vitro ramets sampled (Table 3.1), three samples (8%) contained a total of 449 reads split between six taxa. The only taxa observed in all three samples with reads was Pseudopithomyces rosae. Two of the samples with fungal reads were derived from the same escape shoot and came from ramets housed in the same Magenta box. The third sample with fungal reads was from a WT ramet that had only been micropropagated.

Colonization of greenhouse ramets was limited after 50 days of greenhouse growth. Among the 40 greenhouse samples, 456 fungal reads were split among 28 samples with no sample containing more than 50 reads. The dominant genera were Cladosporium, Pseudopithomyces, and Epicoccum (Figure 3.1). The percentage of samples with reads was higher in greenhouse samples (70%) than in the in vitro samples, although the average number of reads per sample was the same in both sampling efforts (11 reads per sample).

3.4.3 OTU richness increased in subsequent field years

When comparing OTU richness between samples, low sequencing depth among in vitro and greenhouse samples prevented the rarefaction step that was performed on field samples before making contrasts (2,500 reads). When comparing sample richness among WTs sampled from the HIGS common garden in subsequent years, OTU richness increased in year-two (p < 0.005) (Figure 3.1). When comparing WT from the HIGS common garden in year-one to the WT in the established clone bank in year-one, OTU richness was higher on average in the clone bank though the difference was not significant (p = 0.190) (Figure 3.1). No difference was found between year-two HIGS common garden WTs and the established clone bank WTs with mean richness for both groups at approximately 33 OTUS (p = 0.989).
Figure 3.1 Fungal OTU richness of communities detected from lab to field in wild type trees

Bars show the average number of OTUs detected per sample along the propagation pipeline in WT trees, and dots show raw data. Counts are from rarefied data for field samples only. WT trees include both ramets with a history of micropropagation, and non-transgenic escapes. As expected, plants in tissue culture were nearly devoid of fungal symbionts. Greenhouse plants transitioned from lab culture had many more symbionts after 50 days in the greenhouse, but still hardly colonized compared to field samples. Richness increased in field trees sampled over two years (field-1, field-2) (p < 0.005). Clone bank richness was measured at the same time as year-one samples (field_1). No significant differences in richness were detected between field_1 and the clone bank (p = 0.190), or field_2 and the clone bank (p = 0.989).
3.4.4 Dominant taxa detected in the common garden

Reads from field samples were dominated in both years by *Ascomycota* (93.5% in year-one, 92.7% in year-two), followed by *Basidiomycota* (6.4% in year-one, 7.3% in year-two). The majority of reads from within *Ascomycota* were of the class *Dothideomycetes* (99.7% in year-one, 99.3% in year-two). The most abundant genera sampled in both years were *Cladosporium* (49.1% in year-one, 38.1% in year-two), followed by *Epicoccum* (21.5% in year-one, 34.2% in year-two), and *Alternaria* (13.1% in year-one, 11.6% in year-two). *Mycosphaerellaceae* contains *Sphaerulina populicola* and its sister species *Sphaerulina musiva*. Reads from the family *Mycosphaerellaceae* made up 0.4% of reads in year-one and 1.6% in year-two which were present in 5.1% and 56.6% of samples respectively. *Sphaerulina populicola* was detected in only 2.2% of samples in year-one and 5.9% of samples in year-two. As expected, *S. musiva* was not detected at the field site.

3.4.5 No discernible effects due to history of micropropagation

When contrasting communities of field-grown *WTs* with a history of micropropagation to *WTs* that were propagated in a greenhouse from dormant cuttings (*WT-ex vitro*), no differences in community composition were evident in the NMDS ordination (Figure 3.2). Using PERMANOVA, we did not detect an effect of micropropagation on community composition (pseudo-\(f = 1.632, p = 0.125\)). We also did not detect a difference in group dispersions (PERMDISP, \(f = 0.1094, p = 0.762\)), or significant differences in OTU richness (\(p = 0.521\)). Starting sample sizes were equal at the start of the field trial (n=10) however seven of the ex-vitro propagated *WTs* did not survive transplanting. Identical contrasts in year two were not possible because only one ex-vitro *WT* passed sequencing quality filters.
3.4.6 Lack of detectable effects of kanamycin selection and organogenesis

When comparing field samples that originated as non-transgenic escapes (with a history of kanamycin selection and organogenesis) to WTs with only a history of micropropagation, the NMDS ordination did not show differences in community composition (Figure 3.3), and PERMANOVA did not detect significant effects of a history of kanamycin selection and organogenesis in year-one samples (pseudo-$f = 0.957$, $p = 0.416$), or year-two samples (pseudo-$f = 1.417$, $p = 0.224$) (Table S3.1). We also failed to detect differences in group dispersions in year-one (PERMDISP, $f = 2.458$, $p = 0.151$), or year-two (PERMDISP, $f = 1.609$, $p = 0.220$). No significant differences in OTU richness were detected in year-1 ($p = 0.392$), or year-2 ($p = 0.473$).
Figure 3.3 NMDS ordinations of field-grown wild type and non-transgenic escapes propagated from \textit{in vitro} source material

A year-one samples, B year-two samples. Dots are samples units in OTU space. Distance between dots represents dissimilarity of foliar fungal communities. NTE are field samples that originated as non-transgenic escapes (with a history of kanamycin selection and organogenesis. \textit{WT-in vitro} are field samples with only a history of micropropagation.

3.4.7 HIGS effects on non-target fungal communities were not detected

3.4.7.1 All HIGS events and controls

When visualizing communities of \textit{S. musiva}-targeting HIGS lines and controls not targeting \textit{S. musiva} genes with NMDS ordinations, no differences were evident in year-one or year-two (Figure 3.4). When testing for non-target effects of HIGS in year-one, we failed to detect both fixed \textit{S. musiva}-targeting genotype effects (pseudo-\(f = 1.123, p = 0.313\)), or random effects of independent transgenic events nested within genotype (pseudo-\(f = 0.942, p = 0.678\)) (Table 3.2). Similarly, we failed to detect genotype and event effects on non-target fungal communities in the second-year sampling (genotype: pseudo-\(f = 0.766, p = 0.604\); event: pseudo-\(f = 1.037, p = 0.381\)) (Table 3.3). In both years, fertility block and seasonal increase in height were the most useful in explaining variation in community composition (Tables 3.2, 3.3). We did not detect a difference in group dispersions in year-one (PERMDISP, \(f = 0.610, p = 0.482\)), or year-two (PERMDISP, \(f = 1.206, p = 0.288\)). We also were unable to detect differences in OTU richness in year-1 (\(p = 0.506\)) or year-2 (\(p = 0.436\)).
Figure 3.4 NMDS ordinations of HIGS trees and controls

A year-one samples, B year-two samples. Transgenic *Populus trichocarpa* using host-induced gene silencing (HIGS) to target *S. musiva* were grown at a field site without the target pathogen to evaluate the non-target effects of HIGS on foliar fungal endophyte communities. Each dot represents a fungal community from one tree. The distance between dots show dissimilarity between communities. Red indicates samples that use HIGS to target *S. musiva* genes while blue indicates non-*S. musiva* targeting controls (wild type, non-transgenic escapes, and transgenic non-specific HIGS lines). Ellipses show 95% confidence around group centroids.

Table 3.2 Year-one PERMANOVA results for HIGS genotype and event effects on non-target fungal community composition

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Table 3.3 Year-two PERMANOVA results for HIGS genotype and event effects on non-target fungal community composition
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3.4.7.2 High expression events

When restricting analysis of HIGS effects to high HIGS transgene expression lines targeting *S. musiva* *DCL* genes, no differences between transgenic controls targeting *GUS* were evident in the NMDS ordinations in year-one or year-two (Figure 3.5). In year-one, we failed to detect both fixed *S. musiva*-targeting genotype effects (pseudo-f = 1.242, p = 0.285), or random effects of independent transgenic events nested within genotype (pseudo-f = 0.697, p = 0.910) (Table 3.4). Similarly, we failed to detect genotype and event effects on non-target fungal communities in the second-year sampling (genotype: pseudo-f = 0.894, p = 0.462; event: pseudo-f = 0.796, p = 0.757) (Table 3.5). We did not detect a difference in group dispersions in year-one (PERMDISP, f < 0.001, p = 0.995), or year-two (PERMDISP, f = 0.002, p = 0.973). We also did not detect differences in OTU richness in year-1 (p = 0.790) or year-2 (p = 0.533).
Figure 3.5 NMDS ordination of high HIGS transgene expression trees and controls
A year-1 samples, B year-2 samples. Each dot represents a fungal community from one tree. The distance between dots show dissimilarity between communities. Red indicates HIGS samples that target *S. musiva DCL* genes while blue indicates transgenic negative controls targeting *GUS*. Ellipses show 95% confidence around group centroids.

Table 3.4 Year-1 PERMANOVA results for high expression HIGS genotype and event effects on non-target fungal community composition

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Table 3.5 Year-2 PERMANOVA results for high expression HIGS genotype and event effects on non-target fungal community composition

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</table>

3.4.8 HIGS gene target conservation decreases rapidly among antagonist fungal genera
When aligning *CYP51* and *DCL* transcripts for *S. musiva* and its known fungal antagonists over the stretch of sequence targeted by our HIGS constructs, we found that sequence conservation declined rapidly outside of the genus *Sphaerulina* (Figure 3.6). While *Sphaerulina populicola* and *musiva* were not identical, contiguous sequence conservation greater than 21 bp was observed.
Figure 3.6 Alignment of CYP51 transcript sequences over an area targeted by our RNAi construct

The nucleotide similarity in this frame is representative of the nucleotide similarity in other conserved areas of this gene, and of the similarity observed for our other gene targets (DCL). *Sphaerulina populicola* (second from top) is not a known antagonist of *S. musiva* but was included to show the potential for non-target effects in very closely related species. The black bar highlights a consecutive 34 base pair match between *S. populicola* and *S. musiva*. Conversely, consecutive matches greater than 15 base pairs were not observed between *S. musiva* and these known antagonists, and therefore we don’t expect siRNAs derived from our constructs to be capable of silencing homologous genes in non-target genera.

3.4.9 No specific variation in OTUs detected in transgenic plants compared to WT

When looking for effects of HIGS on individual OTUs, we were unable to detect any taxa as differentially abundant compared to control genotypes (Figure S3.1). The estimated effects were all near zero with confidence intervals (±two standard errors) clearly overlapping zero. The family *Mycosphaerellaceae*, containing *S. musiva* and *S. populicola*, whose target sequences for *CYP51* and *DCL* are very similar to that of *S. musiva* (79.9% and 89.8% respectively) (Figure 3.6), also failed to provide any evidence for an effect of HIGS compared to controls (Figure 3.7).
Figure 3.7 HIGS effects on *Mycosphaerellaceae* family OTUs
Twenty-two OTUs were detected in year-one field samples whose taxonomy was assigned to the family containing the target organism *S. musiva*. OTU.86 is the sister species *S. populicola*. Dots show estimated effects of genotypes targeting *Sm-CYP51* genes (A) and *Sm-DCL* genes (B) relative to wild type controls. Error bars are two times the standard error of the estimate. All estimated intervals strongly overlap zero, indicating a lack of detected genotype effects on the abundance of OTUs.

3.5 Discussion

The main goals of this work were: 1) to study the lasting impacts of *in vitro* plant culture, antibiotic selection, and organogenesis associated with genetic transformation on foliar fungal communities under field conditions; and 2) to evaluate the impacts of HIGS on non-target foliar fungi. Neither a history of plant transformation-associated procedures, or
expression of HIGS transgenes targeting specific fungal transcripts, had detectable effects on non-target fungal communities of poplar leaves over two field seasons.

The very low level of fungal reads detected in tissue culture samples suggest our cultures were mostly free of fungal endophytes. Other studies of endophytes in plant tissue culture have focused on bacteria, with few studies including fungal endophytes (Lucero et al., 2011). The lack of fungal endophytes in our study was surprising given that bacterial endophytes are known to endure in cultures despite explant surface sterilization upon initiation into culture (Ulrich et al., 2008; Tamošiūnė et al., 2022). For example, Ulrich et al. (2008) found high densities of endophytic bacteria in cultures of poplar, larch, and spruce that had been sub-cultured for five years, although diversity was low with the majority of bacteria assigned to the genus Paenibacillus.

Fungi are one component of a complex microbiome. The focus on fungi in this study was due to our interest in non-target effects of fungal gene silencing technology. HIGS applications are limited to eukaryotic organisms with functional RNAi pathways. While bacteria should not be directly affected by dsRNA from HIGS plants, it's possible that effects on fungi could influence bacteria indirectly by perturbation of microbial community dynamics. In the case of random mutagenesis of genes controlling plant colonization by plant transformation methods, the effects could extend to all microbes. In this case, disruptions to bacterial community structure could also influence fungal community structure. However, our results suggest that any impacts of transformation on somaclonal variation and HIGS effects were extremely small and far from statistical significance.

Our culture results may have helped to limit fungal colonization in the greenhouse. Our plants were derived from in vitro grown plants that had been acclimated to soil for 21 days then grown in the greenhouse for an additional 50 days. During the time in the greenhouse, we avoided overhead irrigation to limit humid conditions that could be conducive to fungal growth (plants were watered at soil level directly). These results may
not pertain to all greenhouse culture environments; studies in other species and environments with higher fungal loads could give very different results.

Fungal colonization of the field-grown deciduous trees was rapid, but continued into a second growing season. After one season of field growth, common garden WT leaf samples had fungal communities that were as rich as samples of the same genotype from a neighboring and well-established clone bank. With a June planting date, the significant increase in OTU richness between year-one and year-two can likely be explained by year-one leaves missing the majority of spring precipitation that facilitates fungal sporulation and colonization of leaves emerging from bud-break (Barge et al., 2019). However, the comparable OTU richness between common garden samples and clone bank samples suggests there was sufficient colonization to test our hypotheses of non-target effects in both sampling years.

There was not a detectable effect of a history of micropropagation, or of kanamycin treatment, on fungal communities in field-grown trees. While it is possible that there were short-term effects of both micropropagation and antibiotic exposure in our experiment, these effects may not have been detected before sampling at the end of the growing season. Although somaclonal variation can be expected, in poplar its effects on rate of growth and morphology are often minor or transient (Strauss et al., 2016). In species and transformation systems that have more severe physiological and genetic impacts, larger impacts on the fungal endophyte community might occur.

Antibiotic exposure, though with potential mutagenic or epigenetic effects (Bardini et al., 2003), did not cause detectable effects on endophyte levels in field-grown trees. This may be due to its short-term, transient nature, the usually reversible effects of epigenetic modifications, or that the impacts on physiology were too small to significantly affect endophyte cellular habitats. The use of antibiotics other than kanamycin, variations in concentration and duration, and varied responses by other plant species could lead to different outcomes. However, to our knowledge there have been no other studies of antibiotic or transformation effects on endophyte populations.
We were not able to detect an effect of HIGS on non-target fungal communities in the field (Figure 3.4). The potential for non-target effects on fungi is determined by both the sequence of the dsRNA, as well as the presence of fungal endophytes with a capacity for dsRNA uptake. While uptake by target fungi has been demonstrated in numerous studies (Koch and Wassenegger 2021), some fungi are very inefficient at taking up dsRNA, limiting the potential for disease control by HIGS (Kittles et al., 2019; Qiao et al., 2021; Chapter 2). In the case of poor uptake efficiency, the dsRNA sequence would be inconsequential. Nevertheless, uptake efficiencies for the vast majority of fungi remain to be described. Of the fungal OTUs detected in our samples, there were several taxa identified to family and genus level, and one to species, that include species known to take up dsRNA (Mosen, 2022). These included *Verticillium dahliae*, *Fusarium sp.*, *Penicillium sp.*, *Aspergillus sp.*, *Puccinia sp.*, *Melampsora sp.*, and the family *Sclerotiniaceae* of which *Botrytis cinerea*, and *Sclerotinia sclerotiorum* belong.

All HIGS lines were confirmed to have transgene expression; however, as thoroughly discussed in chapter 2, expression was measured in micropropagated cultures only, and the methods we used to estimate dsRNA expression among HIGS transgenic lines may not have been fully reliable measures of the potential for disease resistance or non-target effects. We also found that the dsRNA expressed by our HIGS construct targeting *S. musiva DCL* genes was more effective at silencing target gene expression in vitro than the dsRNA expressed by our HIGS construct targeting *S. musiva CYP51*. For these reasons, we also tested for HIGS effects in a reduced data set that included seven of our highest expressing HIGS events targeting *S. musiva DCL*, yet we still found no effects (Figure 3.5). Despite the lack of detectable HIGS effects, we believe that dsRNA was likely accessible to non-target endophytes that did have a capacity for uptake.

Within populations of transgenic events carrying the same transgene, gene expression and thus associated traits are expected to vary due to position effects, transgene copy number, and the genomic context of random insertion (Kooter et al., 1999, Kohli et al., 2003). All of these factors could have influenced variation in dsRNA expression among HIGS
events, leading to dose dependent effects on non-target endophytes, although none of these were tested directly. Our earlier finding of variable HIGS transgene expression among HIGS events suggested that some events would have a greater potential for target and non-target effects than others (Chapter 2). However, our evaluation of dozens of independent HIGS events did not detect effects of event on non-target fungal communities in the common garden (Tables 3.2, 3.3). Therefore, despite extensive variation in transgene expression, all genotypes in our study population appeared to contain the same communities of foliar fungal endophytes.

Although we found evidence of sufficient sequence homology between *S. musiva* gene targets and homologs in *S. populicola* to suggest a potential for non-target silencing, we failed to detect a non-target HIGS effect on *S. populicola* despite its presence at the field site in both study years. Our earlier finding that *S. musiva* lacks efficient dsRNA uptake (Chapter 2) suggests the same would be true for sister species *S. populicola*, although this was not tested in our study. We also argued that limited dsRNA uptake is the likely reason for our inability to detect resistance to Septoria canker among the same HIGS lines screened in our greenhouse studies (Chapter 2).

HIGS impacts in plant organs other than leaves warrant study. We focused on leaves because: HIGS was targeting a foliar pathogen and we were interested in whether non-target effects would extend to foliar fungi known modify disease (Busby *et al*., 2013; Busby *et al*., 2016). However, in HIGS plants, dsRNA should be accessible to fungal endophytes in all plant tissues including stems and roots. In *Populus*, Cregger *et al*. (2018) have shown that each plant niche contains distinct communities. This additional microbial diversity in non-foliar niches represents an additional reservoir of microbes in which non-target effects of HIGS might be detected. Regardless of the plant tissue, we expect that the effects of HIGS would extend to any fungal endophyte with a capacity for dsRNA uptake, active RNAi, and sufficient sequence conservation of gene targets. For example, in trees, it would be particularly important to evaluate non-target effects among fungal species that form ectomycorrhizal associations with roots.
In conclusion, our findings suggest that HIGS targeting a fungal pathogen, micropropagation, and antibiotic selection and organogenesis associated with plant transformation, all had extremely small, if any, discernable downstream impacts on the foliar microbiomes of *Populus*. In systems where HIGS provides disease resistance, larger impacts would be likely caused by both non-target effects, and indirect effects resulting from the reduction in disease. The extent of such effects will also depend on HIGS construct design and the extent of somaclonal variation imparted. Nonetheless, our results suggest that such impacts may be modest and transient—especially when compared to the many other factors known to have major effects on the microbiome, such as plant genotype, age, growth environment, and management regime.

### 3.6 References


4. Conclusions

The overall goals of this study were to evaluate HIGS as a novel transgenic control strategy for an important disease of poplars, and to evaluate potential impacts that HIGS and plant transformation technologies might have on microbiomes.

In the second chapter of this dissertation, we explored host-induced gene silencing (HIGS) in transgenic poplar as a means to study and control *Sphaerulina musiva*, the cause of Septoria leaf spot and stem canker disease. Greenhouse studies evaluating HIGS transgenic lines targeting either or both *S. musiva* CYP51 and DCL resulted in a lack of statistically detectable resistance. *In vitro* studies evaluating pathogen uptake of dsRNA that is needed for HIGS to function suggested weak or inefficient uptake, and thus also suggested a limited potential for control by HIGS (or direct application of dsRNA as a pesticide, called SIGS or spray-induced gene silencing).

This dissertation broadens our understanding of dsRNA uptake among fungi; *S. musiva* joins a small but growing list of known fungi showing inefficient uptake of exogenous dsRNA and thus limited potential for control by SIGS. Although the number and diversity of fungal pathogens that have been studied with respect to HIGS efficiency is low, it does not appear to be simply predicted by fungal “lifestyle.” For example, among hemibiotrophic fungal pathogens studied to date (e.g., *S. musiva*, *Zymoseptoria tritici*, *Magnaporthe oryzae*), HIGS and SIGS has had variable success. Additional studies in diverse pathosystems are badly needed.

Further study in poplar and other trees about whether HIGS can be a viable strategy for controlling forest pathogens is particularly in need. This is because other methods, such as use of pesticides, are usually uneconomic, and conventional breeding for resistance is often slow and gives complex tradeoffs with respect to breeding progress for other traits. The pathogen's capacity for dsRNA uptake by may be the most important factor that determines HIGS success and should be a priority in research. In poplar for example, it would be of value to know if the biotrophic *Melampsora* rusts could be controlled by HIGS, as effective control of rusts via HIGS has been demonstrated in other
pathosystems. In studies investigating the potential of HIGS control in fungi with biotrophic lifestyles, direct application of dsRNA can be used to evaluate uptake, without the need for plant transformation and resistance tests of transgenic plants.

In chapter three, ITS metabarcoding was used to study foliar fungal communities of transgenic and non-transgenic control trees under field conditions. For the first time, we studied the lasting impacts of \textit{in vitro} plant culture, antibiotic selection and organogenesis associated with genetic transformation. HIGS lines targeting \textit{S. musiva} in a field location absent the target organism were also studied to evaluate impacts of HIGS on non-target fungi. Sampling in tissue culture and greenhouse trees revealed very limited fungal colonization. Once established in the field, fungal colonization was rapid, and continuing to a small extent into the second year. Neither a history of plant transformation-associated procedures, or expression of HIGS transgenes targeting fungal transcripts, had detectable effects on non-target fungal communities over two seasons.

Our studies demonstrate how omics technologies can be used to help evaluate the risks of genetically engineered crops on microbiomes. Empirical, field studies such as we conducted are important because \textit{in silico} predictions on off-target effects of RNAi cannot predict impacts on cryptic endophytic microbes lacking available genomic resources. Moreover, most studies of HIGS against fungi are conducted in laboratories and greenhouses, where plants appear to be nearly devoid of fungal symbionts. It would also be of great interest to conduct comparative studies of microbiome impacts from HIGS vs. that from use of pesticides or conventionally bred crops—where effects are known to sometimes be substantial.

HIGS and SIGS are unlikely to be miracle technologies that make traditional fungal control strategies obsolete. While virus-resistant papaya is a wonderful success story, HIGS and SIGS alone against filamentous pathogens and pests may be much more difficult to achieve. For such diseases, it’s more likely that these technologies can complement existing technologies rather than replace them. HIGS control of western corn rootworm, the only recent commercially deployed HIGS crop, might be telling of what's
to come; RNAi was not very effective alone but instead contributed to overall resistance and durability of pre-existing plant protectants.

Transgene-free SIGS is expected to be commercialized more easily than HIGS, though its success will be challenged by both physical and chemical barriers to uptake by plants and target organisms (e.g., waxy cuticles, environmental RNases), and the economics of repeated pesticide applications. Of course, with any disease control strategy, resistant plants and chemical controls will ultimately select for resistant pathogens. It remains to be seen how pathogens and pests will develop resistance to dsRNA, which could, for example, include selection against dsRNA uptake and thus impair resistance to multiple HIGS-targeted pathogens.

In addition to questions of efficacy, a number of other questions relevant to the use of HIGS in forest trees:

1. Are there likely to be major improvements in HIGS or SIGS technology that might make difficult species and pathosystems, such as we studied, amenable to disease control?
2. Are the benefits of GMO trees, in general, sufficient to overcome social obstacles, including public opinion, economic, market, and regulatory obstacles – which have clearly limited uptake around the world in recent decades. As discussed in chapter 1, these are diverse and long standing.
3. Would the social obstacles be substantially relieved in projects where HIGS protect trees against invasive pathogens that continue to spread and harm native forests?
4. Would the very high specificity of HIGS be an important selling point to the public and thus influential to regulatory evolution? For example, will EPA continue to treat each new HIGS as a novel pesticide with the associated very high regulatory burden?
5. Will there be sufficient advances in transformation technology to enable HIGS to be applied to a diversity of forest tree species and genotypes in a timely and cost-effective manner? Presently, most species and genotypes remain difficult to
transform, making the broad application needed to address the needs of commercial breeding programs difficult to imagine.

Our results suggest that a considerable amount of additional research to better understand the factors that influence HIGS and SIGS efficacy, and the broader social context in which it is applied, are essential.
Appendices
Appendix A Supplementary material for chapter 2

Figure S2.1 Phylogenetic trees of selected *S. musiva* gene targets with homologs from other HIGS studies
Protein sequences were aligned using MAFFT (Katoh and Standley, 2013) and conserved amino acids were used to generate neighbor joining trees. Red underlines show *S. musiva* genes targeted in this study. a Tree showing *S. musiva* cytochrome p450 lanosterol C-α-demethylase (*CYP51*) relationship to orthologs for two fungi whose virulence is reported to have been attenuated by HIGS against *CYP51* genes. b A similar tree for *S. musiva* dicer-like (*DCL*) genes. Sm = *Sphaerulina musiva*, Bc = *Botrytis cinerea*, Mo= *Magnaporthe oryzae*, Fg= *Fusarium graminearum*, Vd= *Verticillium dahliae*. Numbers at tree nodes are bootstrap values.
Figure S2.2 RNAi constructs

**a** *Populus trichocarpa* T61 (SLMB-28-1) was transformed with vectors containing inverted repeat *CaMV35S* promoters (P35S) that drive constitutive expression of sense and antisense transcripts to form dsRNA and are terminated by dual *CaMV35S* terminators (T35S). p9U10-RNAi-Sm-cyp51 targets *S. musiva* cytochrome p450 lanosterol C-α-demethylase (*CYP51*) and two of its homologs while p6U10-RNAi-Sm-DCL targets both *S. musiva* dicer like (*DCL*) genes. p9U10-RNAi-GUS targets *E. coli* beta-glucuronidase (*GUS*) and serves as a non-specific dsRNA control. Selectable markers for kanamycin resistance (*NPTII*) and hygromycin resistance (*HPT*) are driven by *Arabidopsis thaliana* ubiquitin promoter (*UBQ10*) and terminated by *CaMV35S* terminator (T35S).

**b** RNAi inducing sequences from fungal target genes or GUS control.
Figure S2.3 dsRNA sequences used in *in vitro* studies

Synthetic double stranded RNAs *Sm-CYP51* (891 bp), *Sm-DCL* (645 bp), and *GUS* (594 bp) were produced by Greenlight Biosciences and are identical to dsRNA produced by RNAi constructs in figure S2.2 minus the restriction sites added between stacked gene fragments, and the addition of flanking 15 bp ITS sequences (blue). GFP dsRNA (714 bp) was transcribed from a reporter construct containing enhanced green fluorescent protein.
Figure S2.4 Growth of *S. musiva* treated with increasing concentrations of the fungicide tebuconazole in microwell culture

Optical density at 600 nm (OD600) was measured at 4.7 days after treating $10^4$ conidia with increasing amounts of Tebuconazole in a total volume of 0.1 ml potato dextrose broth. Data points represent technical replicates from a single experiment. The minimum inhibitory concentration (MIC) of Tebuconazole for *S. musiva* isolate MN-14—defined as the lowest concentration that prevented visible growth—was 200 nM. Tebuconazole and otherazole fungicides interfere with the protein product of cytochrome P450 lanosterol C14 alpha-demethylase (*CYP51*) genes.
Figure S2.5 Growth of *S. musiva* treated with dsRNA in microwell culture
Optical density at 600 nm (OD600) was measured at 48 hours after treating $10^5$ conidia with increasing amounts of dsRNA in a total volume of 0.2 ml potato dextrose broth. Data points represent results from three independent experiments with OD600 values for treated cells normalized by the OD600 of untreated cells from each experiment. *Sm-CYP51* and *Sm-DCL* dsRNA treatments target housekeeping genes while the control treatment was a non-specific dsRNA (*GUS*). No reductions in growth were found between cells treated with dsRNAs targeting housekeeping genes and cells treated with control dsRNA (one-sided-t-tests, *p*-values shown above brackets).
Figure S2.6 Persistence and stability of dsRNA in culture with *S. musiva* OR its culture filtrate

*S. musiva* conidia (10^5) were cultured in 1% (w/v) potato dextrose broth with 1 µg GUS-dsRNA in a microwell plate. Culture supernatants were collected over time and then run through an agarose gel to evaluate band size and intensities compared to control wells that received only broth and dsRNA. HPI = hours post inoculation. Controls show no sign of dsRNA degradation over the duration of the experiment. 

a dsRNA in culture with *S. musiva* is not detectable after 24 hours. 

b dsRNA incubated with sterile *S. musiva* culture filtrate is not detectable after 8 hours. 

c dsRNA persists when incubated with the same culture filtrate treated with proteinase K.
Figure S2.7 Resistance phenotypes of *S. musiva* susceptible and resistant *Populus* clones in a greenhouse spray inoculation
Plants were sprayed to run-off with a $5 \times 10^5$ conidia/ml suspension. Cankers/cm is the mean number of stem cankers formed three weeks post inoculation divided by plant height at time of inoculation. Canker densities for resistant hybrids 353 and 717 were lower than susceptible *Populus trichocarpa* clone T61 (SLMB-28-1) (Dunnett's test for multiple comparisons, $p$-values above brackets). Error bars show standard deviation.
Figure S2.8 Event resistance compared to WT in 2020 experiment
Estimated differences in mean canker density between events and WT. The hashed horizontal line at zero represents no difference compared to WT. Dots show means and error bars are 95% confidence intervals. The solid horizontal bars at +/- 0.411 show the detectable difference in means using a t-test with the observed variation, n=5, 80% power, and alpha = 0.05. Negative HIGS controls are shown on right (gus).
Mean cankers per centimeter formed three weeks post inoculation for groups of five WT-T61 (SLMB-28-1) plants inoculated with spore suspensions ranging in concentration from $10^2$ to $10^6$ conidia/ml. The purpose of this experiment was to determine the lowest concentration of inoculum that could reliably produce stem cankers on all individuals within a treatment group. At least one canker developed on all plants that received doses of $10^4$/ml and greater. A low inoculum dose was desired to aid in the detection of potential weak resistance phenotypes in continued greenhouse resistance screening efforts. Error bars are standard deviation among trees.
Figure S2.10 Correlation of HIGS transgene and marker gene expression
A Relationship among p9U10-RNAi-Sm-CYP51 events. B Relationship among p6U10-RNAi-Sm-DCL events. Lines are estimated slopes and shading shows 95% confidence.
**Figure S2.11 Event resistance compared to WT in 2022 experiment**

Estimated differences in mean canker density between events and WT. The hashed horizontal line at zero represents no difference compared to WT. Dots show means and error bars are 95% confidence intervals. The solid horizontal bars at +/- 0.119 show the detectable difference in means using a t-test with the observed variation, n=16, 80% power, and alpha = 0.05. Negative HIGS controls and non-transgenic controls are shown on right (gus, NTE).
**Table S2.1 List of primers used**

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Appendix B Supplementary material for chapter 3

Table S3.1 Year-one PERMANOVA results for effect of a history of kanamycin selection and organogenesis
Contrast is between \textit{in vitro} propagated WT (WT-\textit{in vitro}) and non-transgenic escape lines (NTE) by partial sum of squares.

<table>
<thead>
<tr>
<th>Source</th>
<th>df</th>
<th>SS</th>
<th>MS</th>
<th>Pseudo-F</th>
<th>P(perm)</th>
<th>perms</th>
</tr>
</thead>
<tbody>
<tr>
<td>block</td>
<td>3</td>
<td>702.1</td>
<td>234.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>height</td>
<td>1</td>
<td>465.6</td>
<td>465.6</td>
<td>3.60</td>
<td>0.012</td>
<td>9951</td>
</tr>
<tr>
<td>organogenesis</td>
<td>1</td>
<td>123.9</td>
<td>123.9</td>
<td>0.957</td>
<td>0.416</td>
<td>9926</td>
</tr>
<tr>
<td>Res</td>
<td>14</td>
<td>1812.0</td>
<td>129.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>3140.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure S3.1 Effects of S. musiva-targeting genotypes on individual non-target OTUs
OTUs detected in year-one field samples are shown on the x-axis (n=328). Effects were estimated with ALDEx and are shown in red/yellow. Black bars show 95% confidence intervals. No significant effects of HIGS were detected on any of the OTUs.