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Sphaerulina musiva is a fungal pathogen that causes Septoria leaf spot and stem canker on susceptible *Populus* species and hybrids. In this thesis, host and pathogen contributions to Septoria stem canker disease were explored through: 1) a greenhouse study associating Septoria stem canker phenotypes with molecular markers from a genetic linkage map of a *Populus trichocarpa* × *Populus deltoides* F_2 pseudo-backcross population; and 2) the development of a protocol facilitating the genetic transformation of *S. musiva* by *Agrobacterium tumefaciens*. The QTL study localized a single, significant QTL to Linkage Group 16, consistent with currently unpublished reports (Simon et al., unpublished). Additionally, evidence ($p = 0.417$; $p = 0.952$) was obtained that the segregation of resistant-to-susceptible stem canker disease severity was not significantly different from the expected 1:1 ratio, supporting the hypothesis that Septoria stem canker resistance is recessive. Genes within a 1000-Kb window centered on the significant marker on Linkage Group 16 were analyzed for indications of positive

selection (Ka/Ks > 1) and immune-related gene annotations; several candidate genes are discussed. The second chapter describes the development of a protocol for the genetic transformation of *S. musiva* through co-cultivation of *S. musiva* conidia with *A. tumefaciens* cells carrying a binary vector. Using the methods described, transformants were generated. Evidence supports the conclusion that random insertion of a hygromycin resistance cassette into the genome of *S. musiva* isolate MN-14 was successful. However, our attempts at gene-disruption transformation (by homologous recombination into a *nrps1* gene) appear to have failed, though integration of the hygromycin resistance cassette was detected elsewhere in the genome by PCR. Recommendations for further protocol development are discussed. Finally, the implications of the greenhouse QTL study and *S. musiva* transformation are explored in consideration of management implications and future research.

Host and Pathogen Contributions to the *Populus–Sphaerulina* Pathosystem

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

Kyle Thomas Mondron, Author

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Chapter 1: General Introduction

1.1. Importance of *Populus* species

Approximately 30% of the Earth's surface is dominated by forested ecosystems. Forests harbor almost two-thirds of terrestrial biota, and these ecosystems are an important source of the raw materials for lumber, fiber, and lignocellulosic biofuels (Krishnaswamy and Hanson 1999; Balatinecz et al. 2001; Tuskan et al. 2006). World energy demands are predicted to triple from 2015 to 2050 and forest products offer one low carbon emission solution to help meet these demands (Ragauskas et al. 2006; Root and Betts 2015). In the northern hemisphere, *Salix* and *Populus* species, both members of Salicaceae, are well-suited to be potential bioenergy feedstocks. These species and their inter- and intra-specific hybrids exhibit rapid growth, simple vegetative propagation, and abundant genetic variation (Ostry and McNabb 1985). These attributes have led to their widespread planting in windbreaks during the settlement of the Great Plains and, subsequently, their deployment in intensively managed short-rotation plantations (Krupinsky 1989; Kauter et al. 2003).

Populus-based products include pulp, lumber, hardboard, and insulation board (Balatinecz et al. 2001). In addition, the low density, low strength, and high moisture content of *Populus* fiber makes it ideal for composite wood products (Balatinecz et al. 2001). However, the usage of *Populus* wood comes with challenges. Harvested wood

characteristics vary by geographic locale and species (Balatinecz et al. 2001). Although high moisture content is considered beneficial for composite material production, it may prevent uniform drying, cause warping and complicate machine processing (Balatinecz et al. 2001). Moreover, low concentrations of antifungal metabolites can result in susceptibility to discoloration and decay (Balatinecz et al. 2001; Siqueira and Peterson 2003; Preston et al. 2008). Nonetheless, the cultivation of *Populus* species has expanded across global markets, leading to investments in breeding and genomic resources (Wu et al. 1992; Bradshaw and Stettler 1995; Tuskan et al. 2006; Dabros 2008; Feau et al. 2010; Induri et al. 2012; Jonsson et al. 2017).

Populus hybrids are strong candidates for fiber processing and lignocellulosic biofuel development (Balatinecz et al. 2001; Ragauskas et al 2006; Blatner et al. 2015; Bhalla et. al 2016). Hybrid poplars often capitalize on desirable traits from both parent species. For example, fast-growing *Populus trichocarpa* × *Populus deltoides* (T × D) hybrids have greater two-year-old stem volumes and larger leaf areas compared to their parental species (Ridge et al. 1986; Dillen et al. 2009). This phenomenon is referred to as heterosis and is defined as the outperformance of parental species by hybrid progeny. Forest companies have long recognized this potential. In addition to heterosis, hybridization has been used to overcome practical constraints such as rootability. Dormant branches from some *P. deltoides* clones root poorly whereas most *P. trichocarpa* clones root without any difficulty. Crosses between these two species

inherit the rooting ability of the *P. trichocarpa* parent, greatly improving planting success and plantation establishment (Dickmann and Kuzovkina 2014). In 1927, the Oxford Paper Company developed a high-yield hybrid poplar to help meet its fiber demands (Blatner et al. 2015). Since then, these factors have led to the extensive planting of *Populus* F_1 hybrids in commercial plantations around the world (Stettler et al. 1988; Stanton et al. 2010). Of the many hybrid clones available for planting, T × D hybrids are one of the most important to *Populus* cultivation worldwide. These hybrids are considered one of five main commercial taxa developed to meet wood production around the globe (Riemenschneider et al. 2001; Stanton et al. 2010).

1.2. *Populus* and its hybrids: biology

There are approximately thirty species of *Populus* grouped into six distinct taxonomic Sections based on morphological and reproductive differences. These Sections include: Abaso, Turanga, Leucoides, Aigeiros, Tacamahaca, and Populus (Eckenwalder 1996; Dickmann et al. 2002; Isebrands and Richardson 2013). The reproductive phenology of different Sections may limit the potential for hybridization among Sections. For example, Populus-Aigeiros and Populus-Tacamahaca interspecific hybrids are difficult to cross naturally, although techniques involving mentor pollination, chemical treatment of stigmas, and recognition of unilateral incompatibility have led to successful hybridization (Whitecross and Willing 1975; Ronald 1982; Knox et al. 1987). The

differences among Sections extend to leaf morphology as well. Trees in the Section Aigeiros are morphologically diverse with unifacial or bifacial leaves (Dickmann and Kuzovkina 2014). These leaves have either fine or coarse crenations and a deltoid shape (Dickmann and Kuzovkina 2014). In contrast, species in the Section Tacamahaca have bifacial leaves with dentate or crenate leaf margins and the leaves are often narrow and willow-like in shape (Isebrands and Richardson 2013; Dickmann and Kuzovkina 2014).

In general, *Populus* species can reproduce both sexually and asexually. The trees are dioecious with male and female catkins on different trees (Stanton et al. 2014). Pollen is shed in early spring, female catkins are pollinated, and seed is typically dispersed during late spring or early summer, after inflorescence (Boes and Strauss 1994; Broeck et al. 2003). Fertilized seeds contain a cotton-like coating facilitating widespread dispersal prior to germination (Bugala 1973). Obligate outcrossing within the genus *Populus* leads to high amounts of gene flow and a high degree of heterozygosity within individuals and populations (Tuskan et al. 2006).

Populus species have several different modes of asexual reproduction, including rooting of branches and epicormic sprouting from stems and roots (Dickmann and Kuzovkina 2014). *Populus* branches may produce roots at the cut node and lead to the development of apical growth; in a natural environment, branches may become covered with soil and subsequently sprout (Riemenschneider et al. 2001; Stanturf et al. 2001). Commercial and research systems often exploit the rooting of dormant branches (=

cuttings) to quickly develop genetically uniform individuals as planting stock (Castiglione et al. 1993; Louis and Eils 1997; LeBoldus et al. 2010). Epicormic sprouting typically occurs when a *Populus* species is cut near to ground-level during the dormant season (= coppicing) and new shoots grow from the stump (Stanturf and van Oosten 2014). Coppicing often results in a vigorous sprouting of offshoots which is of particular interest in biofuel production systems (Kauter et al. 2003; Bunn 2004; Guidi et al. 2008).

Populus species can form different inter- and intra-specific hybrids (Tyszkiewicz et al. 1968; Rieseberg and Carney 1998). Natural stands of pure *Populus* species often have geographic ranges that overlap, producing swarms of persistent hybrids that may backcross with the pure species, such as the *P. balsamifera* × *P. deltoides* hybrid zone located in Alberta, Canada or the *P. fremontii* × *P. angustifolia* hybrid zone in Utah, U.S.A. (Keim et al. 1989; LeBoldus et al. 2013; Roe et al. 2014). There is evidence that insect herbivory in hybrid zones may be greater than zones composed of pure species due to variation in phenology (Floate et al 1993). The variable phenology results in greater availability of host tissue across a species gradient. Floate *et al.* (1993) observed that *Chrysomela confluens* beetle (Coleoptera: Chrysomelidae) density was correlated with the availability of young, immature leaves in a hybrid zone. This "phenological sink" hypothesis was expanded by LeBoldus *et al.* (2013), who found that leaf emergence date was more variable in hybrid genotypes (σ^2 = 43.8) than pure species (*P. balsamifera:* σ^2= 1.3; *P. deltoides*: σ^2= 1.5).

Some well-known *Populus* hybrid genotypes are the result of anthropogenically-driven intersectional crosses, allowing the desirable traits from different Sections to be captured while maintaining genetic diversity (Dickmann 2001; Riemenschneider et al. 2001). Two commonly crossed sections within the United States are Aigeiros (e.g. *P. deltoides*) and Tacamahaca (e.g. *P. trichocarpa*). Crosses between female *P. trichocarpa* and male *P. deltoides* individuals have been planted widely and used to study the inheritance of complex traits such as height, biomass yield, and resistance to diseases (Wu et al. 1992; Newcombe 1996; Newcombe and Ostry 2001; Induri et al. 2012).

Not all *Populus* species are equally able to hybridize. In addition to the differences in reproductive phenology outlined above, incompatibility is related to both morphological and biochemical differences. For example, obstructive bract hairs protecting the ovary or 'wet' and 'dry' stigma types based upon differential enzyme secretion may prevent hybridization (Villar et al. 1987). Incompatibility may occur both before and after zygote formation (Villar et al. 1987). Pre-zygotic barriers, such as differences in flowering phenology or chemical receptiveness to pollination, may prevent successful crossing (Villar et al. 1987). Synchronizing pollen release and flowering times in a greenhouse may overcome these phenological barriers. However, current solutions to chemical barriers, such as mentor pollen techniques, are considered impractical for adoption by breeding programs (Riemenschneider et al. 2001). Following successful fertilization, there are also several post-zygotic barriers. For example,

premature loss of catkins and flowers, or capsule dehiscence, often results in embryo abortion, although the physiological causes behind post-zygotic barriers are not fully understood (Villar et al. 1987; Raquin et al. 1993; Riemenschneider et al. 2001). Aborted zygote rescue techniques, such as *in vitro* germination or embryo microculture, have been successful and are widely used in breeding programs (Raquin et al. 1993; Riemenschneider et al. 2001).

1.3. *Populus* genetics

Populus trichocarpa was selected to be the first tree sequenced due to its relatively small genome size (ca. 300 Mb), availability of previously mapped quantitative trait loci (QTL), and established transformation techniques (Tuskan et al. 2006). The sequencing of *Populus trichocarpa* in 2006 led to insights into the size and structure of the *Populus* genome (Lescot et al. 2004; Tuskan et al. 2006; Puzey et al. 2012; Wullschleger et al. 2013). Following sequencing, the estimated genome size for the selected genotype of *P. trichocarpa*, Nisqually-1, was 485 Mb, with 1,341,251 SNPs or INDELs located primarily in non-coding regions of the genome (Tuskan et al. 2006). There is on average 2.6 polymorphisms per kilo base pair and approximately 45,600 protein-coding loci (Tuskan et al. 2006). In addition, it is estimated that, approximately 4000 genes may remain to be discovered (Tuskan et al. 2006).

The genome analysis also revealed evidence of three major duplication events in the evolutionary history of *Populus*: 1) an ancient duplication; 2) the Eurosid duplication; and 3) the Salicoid duplication (Tuskan et al. 2006). Due to its early occurrence, the impacts of the ancient duplication remain unclear. However, the Eurosid duplication was a synchronous event affecting 59% of the *Populus* genome and is believed to likely have occurred separately from a similar duplication in *Arabidopsis* (Tuskan et al. 2006). The final duplication event occurred within the last 120 million years. The Salicoid duplication affected both *Salix* and *Populus* and impacted more than 90% of the *Populus* genome. This event resulted in the emergence of approximately 8000 pairs of paralogous genes and a paralogue-dense genome with a complex evolutionary history (Tuskan et al. 2006).

1.4. Diseases of *Populus*

Ostry *et al.* (2014) reported four main pathogens of *Populus* plantations: *Melampsora medusae*, *Marssonina brunnea*, *Sphaerulina musiva*, and *Sphaerulina populicola*. These four pathogens are widely considered to be the most serious pathogens of *Populus* plantations in North America (Ostry and McNabb 1985; Royle and Ostry 1995; Ostry et al. 2014). In each case, disease severity is dependent upon environmental conditions, geographic location, and host genotype.

Melampsora medusae is a fungal rust pathogen native to eastern North America that primarily infects leaves, producing yellow-orange uredinia and urediniospores. This disease reduces growth by impacting the photosynthetic area of leaves and increases susceptibility to other pathogens (Newcombe et al. 2001; Ostry et al. 2014). Since the early 1990s, it has been present in hybrid poplar plantations in the Pacific Northwest (PNW), where many commonly planted *Populus* genotypes may be highly susceptible to infection (Newcombe and Chastagner 1993). *Melampsora medusae* has spread out of its native range and across international borders with several reports in China (Zheng et al. 2019). Management of *Melampsora medusae* relies on planting a diverse selection of resistant genotypes (Ostry et al. 2014).

In one study, resistance to rust was found to be positively correlated with the development of necrotic flecks ($p < 0.001$; Newcombe et al. 1996). Based upon segregation patterns observed over three generations of T (93-968) × D (ILL-129) hybrids, necrotic flecking was found to be under the control of a single, dominant gene conferring partial resistance (Newcombe et al. 1996). A second study on a population of *P. deltoides* progeny formed by crossing a susceptible *P. deltoides* clone (7300501) and resistant *P. deltoides* clone (7302801) found two RAPD markers ($OPG10_{340}$ and $OPZ19_{1800}$) linked to a previously discovered resistance locus (*Lrd*1; Tabor et al. 2000). Due to the co-evolution of *P. deltoides* with *M. medusae*, it is hypothesized that *Lrd*1 may have evolved from selective pressure on the population of *P. deltoides* in the

eastern U.S. (Tabor et al. 2000). Miranda *et al.* (2007) studied the transcriptional response of a population of T × D hybrids to artificial inoculation by *M. medusae* and characterized differential expression of genes between six- and nine- days-post-inoculation. Furthermore, the lack of defense gene upregulation in early timepoints indicate *M. medusae* was capable of avoiding detection despite extensive colonization, and there was a specific accumulation of proanthocyanidins during late infection (Miranda et al. 2007). The role of proanthocyanidins and the flavonoid pathway in *M. medusae* infections is still being elucidated but appears to involve protection against oxidative stress (Mellway and Constabel 2009).

Although primarily a concern in the southeastern Coastal Plains region, *Marssonina brunnea* is a foliar pathogen of *Populus* present across the United States. *Marssonina brunnea* causes small, brownish-black lesions that may coalesce into larger necrotic regions (Newcombe et al. 2001; Ostry et al. 2014). Pycnidia bearing white conidia typically form in these necrotic patches (Newcombe et al. 2001; Ostry et al. 2014). *Populus deltoides* × *P. trichocarpa* and *P. deltoides* × *P. nigra* crosses are particularly susceptible, and lesions may develop on young shoots and petioles, occasionally resulting in defoliation and dieback (Newcombe et al. 2001, Ostry et al. 2014). Seeds may become infected and the movement of non-symptomatic cuttings may result in introductions of *M. brunnea* in new locations (Ostry et al. 2014). Although fungicide applications have been successful in controlling *M. brunnea* outbreaks,

management is primarily focused on reducing primary inoculum sources (e.g. burning leaves before winter) and breeding resistant genotypes for deployment (Ostry et al. 2014).

The molecular mechanisms mediating the interaction between *Marssonina brunnea* and *Populus* have been analyzed in several studies. For example, in a resistant *Populus* hybrid genotype (*P. euramericana*), differential expression of genes putatively related to photosynthesis, metabolism, and defense responses including hydrogen peroxide accumulation were observed (Yuan et al. 2008). In a second study, 1160 genes across the 19 chromosomes of *P. deltoides* (cv. 'Lux') were differentially expressed, including 184 metabolism related genes, 110 signal transduction related genes, 100 transcription and replication related genes, and 90 cell rescue and defense related genes (Zhang et al. 2007). Differential expression of these gene families is typical in comparisons of resistant and susceptible genotypes.

Native to the PNW, *Sphaerulina populicola* Peck commonly causes leaf spot on its native host, *P. trichocarpa* (Zalasky 1978; Newcombe 1996; Feau et al. 2005b; LeBoldus et al. 2009). *Populus* T × D hybrids typically have a disease severity intermediate to their *P. trichocarpa* and *P. deltoides* parents (Newcombe and Bradshaw 1996). In a two-year study of *S. populicola* leaf spot, a single significant QTL located on Linkage group X was found to explain 44.8% and 26.2% of the phenotypic variance each

year. Two additional QTL were located on Linkage groups A and M but were not consistent across all years of the study.

Outbreaks of the fungal pathogen *Sphaerulina musiva* (Peck) Quaedvl., Verkley, & Crous (syn. = *Septoria musiva*) in exotic *Populus* and hybrid plantations result in severe leaf spot and stem infections (Bier 1939; Spielman 1986; Ostry 1987; Callan et al. 2007). *Sphaerulina musiva* is closely related to *S. populicola* but is native to northeastern North America where it causes leaf spot on its sympatric host *P. deltoides* (Waterman 1954). Native stands of *P. deltoides* serve as a source of inoculum for neighboring plantations, threatening susceptible Tacamahaca species and hybrids with outbreaks of severe stem canker and leaf spot (Ostry 1987; Luley and McNabb 1989; Newcombe and Ostry 2001). In particular, T × D F_1 hybrids are considered highly susceptible to both leaf spot and stem canker (Ostry and McNabb 1985). Leaf spots impact the photosynthetic potential of leaves, reducing growth rates and resulting in premature defoliation (Bier 1939; Cooper and Filer 1976). Cankers form on stems and branches, compromising structural integrity and causing tree mortality (Zalasky 1978; Ostry and McNabb 1985).

The most susceptible *Populus* genotypes and hybrid clones could see biomass losses from *S. musiva* greater than 60% (McNabb et al. 1982). Several authors have gone so far as to suggest that “clones with even slight damage from cankers cannot be recommended” (Ostry and McNabb 1985). *Sphaerulina musiva* is considered to have prevented the development of hybrid poplar plantations in the central United States by

causing high rates of mortality and commercial failures (Ostry and McNabb 1985; Strobl and Fraser 1989). For example, in a mixed-hybrid plantation on a 15-year rotation, 86% of the trees had Septoria stem canker symptoms five years after planting, and 69% of the trees had broken tops seven years after planting (Ostry et al. 1989). The authors noted that the vast majority of the plantation was expected to die prior to harvest (Ostry et al. 1989). In the Tennessee Valley, all ten genotypes planted at the Norris arboretum were infected with *Sphaerulina musiva*; three of the genotypes were completely killed (Blow 1948).

Understanding the source of resistance to Septoria stem canker in *P. deltoides* and how resistance is inherited by progeny of different genetic backgrounds can assist breeding programs in the development of a wider array of resistant clones. Notably, the commercially desirable *P. trichocarpa* (93-968) × *P. deltoides* (53-242) F_1 hybrid population was found to be entirely susceptible in the 2001 study by Newcombe and Ostry, and 100% (26/26) of tested ramets were susceptible to Septoria stem canker (Newcombe and Ostry 2001). LeBoldus *et al.* (2009) observed a 100% infection frequency in a field study of *S. musiva* stem canker on *P. balsamifera* (Section Tacamahaca). Due to these dramatic impacts, *S. musiva* is largely considered the most significant pathogen of *Populus* plantations in North America, with significant economic and ecological importance (Bier 1939; Spielman 1986; Ostry 1987).

1.5. Infection biology and epidemiology of *S. musiva*

Sphaerulina musiva is a Dothideomycete in the Ascomycotina with a life cycle dominated by a haploid asexual stage (Dhillon et al. 2019). This stage produces pycnidia, spore-forming structures that have their base and sides lined with conidia (Waterman 1954; Feau et al. 2005a). Pycnidia develop within symptomatic tissue, including leaf spots and cankers, throughout the growing season (Bier 1939; Waterman 1954; Feau et al. 2005a). Under moist conditions, conidia are dispersed in pinkish tendrils (Qin and LeBoldus 2014). Individual conidia are hyaline with 1-4 septations and have a width ranging from 2.0-3.2 microns and length ranging from 20-52 microns (Bier 1939; Filer et al. 1971). The sexual stage of this fungus is diploid and is typically seen as overwintering pseudothecia on leaf litter (Feau et al. 2005a). The flask-shaped pseudothecia contain asci, each composed of eight cylindrical, uniseptate ascospores, which are considered the primary inoculum responsible for early spring infections (Bier 1939). Ascospore release is correlated with bud-break in early Spring (Bier 1939). *S. musiva* is heterothallic with two mating type idiomorphs (MAT1-1 and MAT1-2). Sexual and asexual reproduction have been reported in population genetic analyses of the fungus (Feau et al. 2005a; Sakalidis et al. 2016; Tabima et al., 2019).

Using a series of Random Amplified Polymorphic DNA (RAPD) markers, one study found evidence of sexual reproduction in *S. musiva* populations, emphasizing the likelihood that sexual recombination plays a role in field infections (Feau et al. 2005a).

However, identical haplotypes were recovered more often than expected from a randomly mating population, indicating that a clonal reproductive system remained the primary method for reproduction (Feau et al. 2005a). Comparing intra-leaf, between-leaf, between-tree, within-tree, and among-location variability, revealed that more than 90% of the genetic variability was distributed within samples from the same tree. Different leaves were often colonized by different haplotypes. On a broader scale, identical haplotypes found within a limited range helped support a hypothesis of localized, asexual reproduction (Feau et al. 2005a). If ascospores are widely disseminated, gene flow would have a large role in genetic structure and individuals between populations would be genetically similar. However, populations were more genetically diverse than would be expected, indicating that the role of ascospore dispersal may be limited (Feau et al. 2005a). More recently, a total of 122 isolates of *S. musiva* collected from diseased trees across North America revealed 120,016 bi-allelic single nucleotide polymorphisms (SNPs) which grouped isolates into three distinct genetic clusters (SE-US, US-CANADA, and BC2; Tabima et al. 2019). This led to the discovery of a significant history of recombination ($p < 0.001$) and evidence of differences in cankers per centimeter and disease severity score by genetic cluster ($p < 0.001$) and isolate ($p < 0.001$; Tabima et al. 2019).

Bier speculated in the late 1930s that lenticels, stomata, and petioles may serve as points-of-entry for both conidia and ascospores (Bier 1939). Several authors have

reported infections on new growth without evidence of wounds, supporting Bier's hypothesis (Filer et al. 1971; Zalasky 1978; LeBoldus et al. 2010). Qin and LeBoldus (2014) characterized conidia entering stem tissue through lenticels and other natural openings. Abraham *et al.* (2019) reported similar observations on inoculated leaves where germ tubes penetrated host tissue through both stomata and directly through the unwounded epidermis. In both cases, following penetration, intercellular hyphae were observed to develop extensively in susceptible host tissue, leading to stem canker and leaf spot symptoms within three weeks of inoculation (Weiland and Stanosz 2007; Qin and LeBoldus 2014; Abraham et al. 2019).

Beginning in the late 1990s, *S. musiva* has been reported outside its native range in North America and around the world. These reports include Brazil, Argentina, and regions within Asia (Sivanesan 1990; Maxwell et al. 1997; Callan et al. 2007; Santos et al. 2010). Since then, the native *P. trichocarpa* stands along the western coasts of North America have been considered at-risk for infection. As its range expands, *S. musiva* threatens more than 440 km^2 of plantations in Canada and the United States (Belgium 2012). It is currently hypothesized that *S. musiva* has likely been introduced to western North America in two separate events with unique origins in West Virginia and Pennsylvania (Tabima et al. 2019). Introductions are likely the result of anthropogenic movement of infected planting stock (Sakalidis et al. 2016, Tabima et al. 2019). Given the evidence of asymptomatic *S. musiva* infections detectable via qPCR and similar

human-driven pathogen dispersal in other systems (e.g. *Magnaporthe oryzae*), the role of anthropogenically-facilitated spread is highly likely.

1.6. Management of *S. musiva* diseases

The management of *S. musiva* has focused on chemical, biological, and cultural control. Fungicides, such as benomyl 50% W.P. (Wettable Powder), chlorothalonil 75% W.P., captafol 80% W.P., and mancozeb 80 W.P. + Spreader Sticker, have been effective at controlling *Septoria* leaf spot and stem canker when applied 3-5 times during the growing season (Ostry 1987). Bimonthly applications of benomyl reduced canker incidence from 3.2 cankers/stool to 1.3 cankers/stool on the susceptible *P. trichocarpa* × *P. nigra* hybrid genotype (*P.* × *euramericana,* DN28; Ostry 1987). Bimonthly applications of chlorothalonil, captafol, and mancozeb resulted in similar reductions in canker incidence (Ostry 1987). An earlier study examined the efficacy of four applications of benomyl 50% W.P. at 10-day intervals (Carbon 1972). This regime resulted in a reduction in leaf senescence of approximately 19.6% relative to the controls (Carbon 1972). Although effective, over the course of a 10-20-year rotation, the cost of repeated applications of fungicide is prohibitive (McNabb et al. 1982; Ostry 1987). In addition, the risk of fungicide resistance and adverse environmental impacts could further limit the long-term success of chemical control (Fernández-Ortuño et al. 2008). Nevertheless, one

potential strategy may be the treatment of cuttings and other planting stock from nurseries to limit the risk of pathogen dispersal (McNabb et al. 1982; Ostry 1987).

Largely a natural, low-impact alternative to agrochemical approaches, biological control (or "biocontrol") involves using microbial and insect antagonists to reduce the populations of specific pests or pathogens (Pal and Gardener 2006). Various strains of *Streptomyces* bacteria have been assessed as potential biocontrol agents against *S. musiva* in plantations of *Populus* (Gyenis et al. 2003). Reduced leaf spot was observed in both *Streptomyces* single-strain and *Streptomyces* mixed-strain applications (Gyenis et al. 2003). Following application, reductions in leaf disease severity scores ranged from 1.1-1.3 for single *Streptomyces* strain products and 1.0-1.5 for mixed strains relative to controls, on a scale of 0.0-8.0 (Gyenis et al. 2003). The efficacy of *Streptomyces* to prevent the development of stem canker symptoms was not evaluated (Gyenis et al. 2003). Similar to fungicides, many applications of the biocontrol agent may be necessary throughout a rotation for effective control. As a result, biocontrol cannot attenuate the threat of *S. musiva* stem canker at this time.

Cultural control aims to prevent plants from coming into contact with a pathogen by altering the environmental conditions, eradicating the pathogen, or reducing the amount of the pathogen in an area. These treatments are typically achieved by adjusting conventional management decisions (Selman 1941; Ostry 1987; Jacobsen 1997). For example, shorter rotation times may help limit the impact of *S.*

musiva on wood quality. However, given that *S. musiva* stem cankers can girdle trees and cause stem breakage in as little as 4 years after planting, this approach is unlikely to be effective (Schreiner 1972; Ostry and McNabb 1983; Ostry 1987). A second approach tested was the elimination of primary inoculum by burying infected leaf debris in the Spring prior to leaf flush (Ostry 1987). Inoculum spread from neighboring plots or the surrounding forest resulted in no disease control. The main conclusion from these studies was that cultural control was largely ineffective. Given the limited success of chemical, biological, and cultural control, disease resistance is the only viable option for the management of Septoria leaf spot and stem canker (Ostry and McNabb 1985; Feau et al. 2010).

1.7. Disease resistance in the *S. musiva–Populus* pathosystem

1.7.1. Variation in the host population.

A wide array of *Populus* species and hybrids have been evaluated for resistance to leaf spot and stem canker (Ostry and McNabb 1985, 1986; Spielman 1986; Newcombe and Ostry 2001). Taxonomic patterns in susceptibility have emerged. Species in the Section Tacamahaca are typically susceptible to stem canker whereas species in the Section Aigeiros are only susceptible to leaf infections (Newcombe and Ostry 2001). Hybrid poplar clones between species from Tacamahaca and Aigeiros, such as *P. trichocarpa* × *P. deltoides*, are susceptible to both leaf spot and stem canker disease. Differences in

susceptibility among clones have been reported in both the field and artificial inoculations conducted in the greenhouse (Ostry and McNabb 1985, 1986; Spielman 1986; Newcombe and Ostry 2001; Weiland et al. 2003; LeBoldus et al. 2008, 2009; Dunnell et al. 2016). In general, the majority of the variation in resistance can be attributed to variation among genotypes (LeBoldus et al. 2008, 2009; Dunnell et al. 2016). Broadly, the ranking of the most susceptible and resistant host genotypes remains stable regardless of the environment where the trees are planted or the population of *S. musiva* the host is exposed to (Strobl and Fraser 1989; Krupinsky 1989; Weiland et al. 2003; Qin et al. 2014).

Given that disease resistance is widely considered the best approach for managing Septoria leaf spot and stem canker, there are relatively few studies examining the mechanisms of resistance or how resistance is inherited. A single published study evaluated the segregation of resistance in a backcross population developed by crossing a T x D female hybrid genotype [53-246; produced by crossing a female *P. trichocarpa* (93-968) with a male *P. deltoides* (ILL-129)] with the pollen parent *P. deltoides* (ILL-129; Newcombe and Ostry 2001). The F_1 progeny were all susceptible to Septoria leaf spot and stem canker and the TD × TD F_2 progeny were expected to segregate in a 1:3 ratio of resistant to susceptible (Newcombe and Ostry 2001). Likewise, the TD × D backcross progeny were expected to segregate in a 1:1 ratio of resistant to susceptible

(Newcombe and Ostry 2001). The authors hypothesized that resistance was inherited in a recessive manner (Newcombe and Ostry 2001).

More recently a similar, pseudo-backcross population (52-124) produced by crossing a T x D female hybrid genotype [52-225; produced by crossing a female *P. trichocarpa* (93-968) with a male *P. deltoides* (ILL-101)] with a genetically similar *P. deltoides* male (D124) revealed a significant QTL correlated to *S. musiva* stem canker severity at a field site in West Virginia. This QTL was localized to Linkage Group 16 of the *Populus* genome, at approximately 60.81 cM (Muchero et al. 2015; Simon et al., unpublished). In both cases, there were a large number of potential disease escapes and as such, conducting an experiment under controlled conditions was suggested as way to formally test this hypothesis (Newcombe and Ostry (2001).

The sequencing of the *P. trichocarpa* genome and the re-sequencing of 545 genotypes of *P. trichocarpa* in the development of a genome wide association mapping population has drastically improved the tools available to study the molecular mechanisms of resistance to *S. musiva* (Tuskan et al. 2006; Evans et al. 2014; Muchero et al. 2018). Liang *et al.* (2014) found 36 differentially expressed genes which were consistent in two resistant hybrid genotypes (DN34, *P. deltoides* × *P. nigra*; NM6, *P. nigra* × *P. maximowiczii*). Genes upregulated in resistant genotypes contained functional annotations related to protein fate, cell wall structure, and stress responsiveness, including a glycosyltransferase required for pathogen resistance (Potri.006G272600). In

contrast, two susceptible genotypes (DN164, *P. deltoides* × *P. nigra*; NC11505, *P. maximowiczii* × *P. trichocarpa*) had upregulation of genes with functional annotations including phospholipases and an elicitor-activated gene product (Liang et al. 2014). Similarly, Abraham *et al.* (2019) saw transcriptional upregulation in a moderately resistant genotype (DN99; *P. deltoides* × *P. nigra*) of cell wall modification genes (such as pectin lyase, pectin methyl esterase, and lignin biosynthesis enzymes), antioxidant-encoding genes (such as catalases and peroxidases), and PR family proteins (such as PR1 and thaumatin). Studies found evidence that the production of reactive oxygen species may be important to the response of *Populus* to infection by *S. musiva* (Liang et al. 2014; Abraham et al. 2019).

More recently, transcriptional changes have been linked to polymorphisms in candidate genes. For example, a Genome-Wide Association Study (GWAS) leveraging the *P. trichocarpa* resource found single nucleotide polymorphisms (SNPs) that were associated with susceptible and/or resistant responses (Muchero et al. 2018). Two receptor-like proteins (RLP1, Potri.005G012100; RLP2, Potri.009G036300) and a L-type lectin receptor-like protein kinase (Potri.003G028200) were associated with resistance; a G-type lectin receptor-like protein kinase was associated with susceptibility (Potri.005G018000; Muchero et al. 2018). However, the role of these genes in the resistance of *Populus* hybrids to Septoria stem canker requires further validation.

1.7.2. Variation in pathogen aggressiveness

Differences in aggressiveness among isolates have been reported in several studies (Krupinsky 1989; Maxwell et al. 1997; LeBoldus et al. 2008; Dunnell et al. 2016). For example, Krupinsky (1989) found differences in leaf spot severity among twenty-eight isolates of *S. musiva* when five genotypes of *Populus* were inoculated. The observed variation in aggressiveness among isolates was similar within and among geographic collection locations, suggesting that a variety of the most aggressive isolates from one geographic location are sufficient to evaluate host resistance (Krupinsky 1989). Feau *et al.* (2005a) found similar patterns in genetic diversity using Random Amplified Polymorphic DNA (RAPD) when comparing isolates collected at multiple geographic scales.

Several studies have evaluated the variation in aggressiveness among isolates in terms of stem canker severity. LeBoldus *et al.* (2008) found that isolate effects explained 15% of the variance in a study of 19 isolates infecting 14 different *Populus* clones. Similarly, another study by LeBoldus *et al.* (2009) saw 14% of the variance explained by an isolate effect, when 10 *Populus* clones were inoculated by 7 isolates of *S. musiva* collected from Quebec and Alberta. An isolate effect was found to explain approximately 3.2% of the variation in disease severity scores in an experiment in which 47 genotypes of *P. nigra* were inoculated with 6 isolates of *S. musiva* (Dunnell et al. 2016). Tabima *et al.* (2019) found differences in disease severity among three genetic

clusters of *S. musiva* described in that study. Although the effect of host genotype appears to explain more variation, different isolates of *S. musiva* appear to differ in aggressiveness.

1.7.3. Variation in the host-pathogen interaction

To develop an understanding of which host genotypes will perform well in different environments and in the presence of different isolates of *S. musiva*, the importance of genotype-by-isolate interaction must be characterized. Evidence for a genotype-by-isolate interaction in *S. musiva* stem canker has been mixed. For example, LeBoldus *et al.* (2008) found a significant genotype-by-isolate effect ($p = 0.03$), although the majority of the variation was explained by host genotype (LeBoldus et al. 2008). Tabima *et al.* (2019), in their population genetics study of 122 isolates of *S. musiva,* found a significant interaction between the host genotype and pathogen genetic cluster. In contrast, Abraham *et al.* (2018) found no evidence of genotype-by-isolate interactions in either disease severity ratings or qPCR-facilitated analysis of resistance ($p = 0.868$ and $= 0.694$, respectively). Overall, the role of a clone-by-isolate interaction in this pathosystem is surpassed by the effects of the host genotype; however, further investigation is warranted.

1.7.4. Environmental factors

The role of the environment in plant pathology makes up a third of the disease triangle paradigm. This paradigm suggests that interactions between the host, pathogen, and environment impact disease incidence and severity (Francl 2001). An analysis of water stress effects on *S. musiva* stem canker by Maxwell *et al.* (1997) found that water stress was significantly associated with the percentage of poplar stem girdled in one of two stem inoculation studies (Study 1, 80 days post-inoculation, $p = 0.0842$; Study 2, 75 days post-inoculation, $p = 0.0001$), although the canker length response was significantly associated with water stress in both experiments ($p = 0.002$ and $p = 0.0005$, respectively). Water stress impacts were revisited by LeBoldus *et al.* (2007) where water stress did not significantly affect the disease severity phenotype ($p = 0.258$), and the majority of variance (89%) was explained by the genotype alone. When resistance screening is performed in a greenhouse environment, the effects of environment appear to be limited and unlikely to impact the resistance phenotype compared to host genotype (Weiland et al. 2003, LeBoldus et al. 2007; Qin et al. 2014).

1.8. Plant immunity, genetic markers, linkage maps, and quantitative trait loci

Unlike animals, which have both innate and adaptive immune systems, plants rely on innate immunity and systemic signaling to defend themselves from pathogen attack (Jones and Dangl 2006; Iriti and Faoro 2007; Kushalappa et al. 2016). A common feature

of nearly all plant immune systems is the ability to recognize broadly conserved, molecular patterns (Kushalappa et al. 2016). This includes a broad range of microbe-associated molecular patterns (MAMPs), pathogen-associated molecular patterns (PAMPs), and damage-associated molecular patterns (DAMPs; Bittel and Robatzek 2007; Henry et al. 2012; Macho and Zipfel 2014). These molecular patterns are detected by pattern recognition receptors (PRRs) typically anchored in the plasma membrane with an extracellular domain in the plant apoplast. Recognition of MAMPs, PAMPs, and/or DAMPs is the first and most general layer of plant defense, triggering cellular responses and downstream signaling (Kushalappa et al. 2016).

Jones and Dangl (2006) described the so-called “Zig-Zag model” to explain how this general layer of basal plant defense, PAMP-triggered immunity (PTI), could evolve into a more advanced plant immune system. They describe plant pathogens and their hosts as caught in a “molecular arms race” in which both parties co-evolve genes that mediate the interaction between the two organisms. In order to circumvent the PTI response, pathogens have evolved small secreted proteins, called effectors, which exploit a myriad of mechanisms to delay or suppress PTI. Although effector function is poorly understood in many cases, plants are believed to have evolved R-genes producing specialized R-proteins that recognize pathogen effectors. Recognition of effectors by cognate R-genes triggers localized programmed cell death called the hypersensitive response. This pathway is also known as effector-triggered immunity

(ETI; Jones and Dangl 2006; Kushalappa et al. 2016; Künstler et al. 2016). The Zig-Zag model proposes that pathogens then evolve new effectors and lose compromised effectors, perpetuating the arms race (Jones and Dangl 2006).

The advent of modern molecular markers, such as amplified fragment length polymorphisms (AFLPs), restriction-fragment length polymorphisms (RFLPs), single-nucleotide polymorphisms (SNPs), and insertion-deletion mutations (INDELs) has improved our ability to identify the gene(s) underlying a plant phenotype (Lander and Botstein 1989; Liu et al. 2001; Huang and Röder 2004; Collard et al. 2005; Kushalappa et al. 2016). These markers follow well-characterized patterns of inheritance and if they are spread across a genome can be used to identify locus/loci associated with a specific phenotype in a process called genetic mapping. This process assumes that in order for two markers (or loci) located on the same chromosome to be inherited together they need to be in close proximity (Slatkin 2008; Boopathi 2013). By studying the offspring from a specific cross, one can derive a recombination frequency, and use this to compute the relative distance among loci. After ordering the markers relative to one another, a linkage map is produced. A linkage map is a statistical map of predicted chromosomes (called “linkage groups”), the relative position of markers on the chromosomes, and the allele status of each individual in the population at each marker (Suiter et al. 1983; Lander et al. 1987; Stam 1993). Once a linkage map is available, it can be used to make genotype-phenotype associations based on the pattern of inheritance

observed between the marker(s) and a specific phenotype (Bradshaw and Stettler 1995; Young 1996; Broman et al. 2003; Collard et al. 2005).

When a quantitative trait is correlated to a genotype using marker associations, they are called quantitative trait loci (QTL; Miles and Wayne 2008). The likelihood of associations between a certain marker and a phenotype (i.e. disease resistance) can be estimated by calculating the odds that a gene and a marker are located close to each other in a genome (Nyholt 2000; Collard et al. 2005). The strength of the association between a marker and a phenotype is called the LOD (logarithm of the odds) score. The larger the LOD score, the stronger the statistical association between the marker and the trait (Young 1996; Broman et al. 2003; Collard et al. 2004). Despite the power of QTL mapping approaches, associations depend upon proximity of the locus controlling the trait to the segregating markers. As a result, there can be a large genetic interval the gene(s) controlling the trait could be found within (Kearsey and Farquhar 1998). There are several approaches used to examine the genetic interval around the significant markers. These approaches include: 1) all genes between the significant marker and the distance before LOD is reduced by 1 in both directions; and 2) genes within a 1-Mb window of the peak (Yu et al. 2005; Stridh et al. 2010). To date, QTL mapping has been instrumental in the improvement of many important crops (Bradshaw and Stettler 1995; Yin et al. 2003; Concibido et al. 2004; Induri et al. 2012).

In agriculture, a backcross population is commonly used to detect QTL linked to disease phenotypes and involves crossing a resistant and a susceptible species to create a population of heterozygous F_1 progeny, one of which is then crossed with one of the original parent genotypes to produce the backcross population (Collard et al. 2005). However, species that do not tolerate in-breeding, including *Populus*, may not produce sufficient progeny for mapping if backcrossed with a parent genotype. An alternative approach uses a genetically similar genotype of the same species to study the segregation patterns of inherited alleles. This is called a pseudo-backcross and has been used to map QTL associated with disease resistance and other traits in *Populus* (Newcombe and Ostry 2001; Muchero et al. 2015; Simon *et al.,* unpublished).

1.9. Candidate gene selection criteria

Following identification of a QTL, identifying the gene(s) controlling the phenotype can still be extremely difficult given the large number of potential candidate genes encompassed by the peak. Indicators of positive selection, such as the Ka/Ks ratio, are used to identify genes under selective pressure (Fay et al. 2001). Here, Ka represents the rate of non-synonymous mutations between homologous protein sequences, and Ks is the rate of synonymous mutations between homologous protein sequences (Nekrutenko et al. 2002). Non-synonymous mutations often lead to functional differences in the resulting gene product (Kelly and Price 2000). Genes with a high Ka/Ks

ratio may have undergone positive selection (Fay et al. 2001; Xing and Lee 2005; Zhang et al. 2006). For example, if a gene has a disease resistance function, it would provide a strong evolutionary advantage and may reveal this history as a large Ka/Ks ratio. This pattern is typical of leucine-rich repeat (LRR) domains which have well characterized roles in pathogen recognition (Holt III et al. 2000). In contrast, if Ka is approximately equal to Ks then the locus is considered to be under neutral selection. Finally, if Ks is larger than Ka the locus is under negative (or purifying) selection, where mutations are selected against in a population (Zhang et al. 2006). For example, Warren *et al.* (2010) found that genes encoding important ribosomal structural molecules were likely to have lower rates of non-synonymous mutation when compared to genes related to disease resistance.

A second approach which can be used to reduce the number of candidate loci in a significant QTL are gene annotations. These models suggest a gene's predicted function by analyzing DNA or amino acid sequences and comparing them to homologous genes in another organism, typically *Arabidopsis* in plants (Ramírez-Carvajal et al. 2008). These descriptors, along with domain classifiers, such as GO (gene ontology) and PANTHER (Protein ANalysis THrough Evolutionary Relationships), identify proteins whose domains appear to have a function related to a specific phenotype (Young et al. 2010; Mi et al. 2018). For example, in the case of disease resistance, designations include leucine-rich repeats (LRRs), nucleotide-binding sites (NBS),

receptor-like kinases (RLKs), receptor-like proteins (RLPs), and others (Jones and Jones 1997; Ferreira et al. 2007; Goff and Ramonell 2007). However, it is important to keep in mind that gene annotations are only predictions based upon sequence similarities to similar organisms; they are not replacements for empirical evidence and may be inaccurate (Lin et al. 2008). One way to validate the role of candidate genes is through the use of transgenic approaches.

1.10. *Agrobacterium tumefaciens*-mediated transformation of filamentous fungi

Agrobacterium tumefaciens was originally discovered as a plant pathogenic bacterium capable of inserting its plasmid DNA into a host, causing crown gall tumors (Păcurar et al. 2011). Successful transformation required the Ti-plasmid (tumor-inducing plasmid), which contained the T-DNA and *vir* region (Watson et al. 1975; Yadav et al. 1982; Barker et al. 1983; Păcurar et al. 2011). The T-DNA is the *A. tumefaciens* DNA which induces crown gall development in susceptible hosts and the *vir* region consists of several genes responsible for making the proteins necessary for formation of the Type 4 Secretion System (T4SS) and T-DNA insertion (Păcurar et al. 2011). Researchers realized that rather than inserting crown gall-causing DNA into a plant genome, *A. tumefaciens* could be used as an engineering tool to insert other DNA sequences. *Agrobacterium tumefaciens* has been used to transform plants, fungi, and other organisms in order to validate the function of genes (Nam et al. 1999; Valentine 2003; Homrich et al. 2012).

The integration of the gall-causing T-DNA in pathogenic *A. tumefaciens* occurred randomly in the host genome (Păcurar et al. 2011). However, homologous recombination can be exploited to result in targeted DNA insertion or replacement events (Paz et al. 2011; Wang et al. 2016). If there is homology between the T-DNA and the host genome, an exogenous cassette (the engineered T-DNA) can replace the homologous loci in the host (Foster et al. 2014; Idnurm et al. 2017). Incorporating resistance genes (such as hygromycin resistance) into the homologously-designed T-DNA can allow for efficient selection of transformants (Dobinson et al. 2004; Foster et al. 2014). Evidence exists that the same technique can disrupt fungal hosts as well (Dobinson et al. 2004; Sugui et al. 2005). The successful transformation of the poplar pathogenic ascomycete *S. musiva* revealed an opportunity to disrupt potentially important genes within a plant pathogenic fungus to study how potential effectors or pathogenic proteins may contribute to symptoms in *Populus* (Foster et al. 2014). However, before pathogenic genes can be disrupted and the impact of their loss related to disease phenotypes, a protocol must be developed.

1.11. Study rationale and research objectives

The overall goal of this thesis is to develop a better understanding of host-parasite interactions in the *S. musiva–Populus* pathosystem. To this end, two studies were conducted. In the first study, a pseudo-backcross population of T × D hybrids was

inoculated with *S. musiva* under controlled conditions. The specific objectives were to: 1) test the hypothesis that resistance is recessive and inherited form the *P. deltoides* parent; 2) identify other potential QTL associated with resistance; and 3) develop a list of candidate genes which could be validated in future experiments. In the second study, a protocol using *A. tumefaciens*-mediated transformation (ATMT) of *S. musiva* was adapted from Khang *et al.* (2006) and Foster *et al.* (2014). The specific objectives were to transform *S. musiva* with two different binary vectors: 1) one designed to disrupt a specific gene; and 2) a second designed to randomly disrupt genes within the *S. musiva* genome.

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Chapter 2: QTL Involved in Septoria Stem Canker on Poplar Hybrids

2.1. Introduction

Trees belonging to the genus *Populus* provide raw materials for the fiber and bioenergy industries (Rose et al. 1981; Balatinecz et al. 2001). Furthermore, *Populus* species are widely distributed and ecologically important in the northern hemisphere (Dickmann 2001; Isebrands and Karnosky 2001). Due to a predisposition for hybridization, *Populus* hybrids can be bred for desirable traits such as increased biomass yield and fast root development on dormant branches (Stout and Schreiner 1933; Allwright and Taylor 2016). As such, many genetically identical hybrid poplars may be propagated as clonal stock (Dickmann 2001; Riemenschneider et al. 2001). However, despite *Populus trichocarpa* being a model species with a well-developed reference genome, cultivation of *Populus* is impeded by disease (Ostry and McNabb 1983; Newcombe et al. 2001; Tuskan et al. 2006; Wullschleger et al. 2013; Ostry et al. 2014).

Generally considered the most severe pathogen of *Populus* species, the ascomycete *Sphaerulina musiva* (Peck) Quaedvl., Verkley & Crous (syn. = *Septoria musiva* Peck) causes leaf spot in the northeastern United States on its sympatric host *Populus deltoides* (Feau et al. 2010). This pathogen causes branch and stem cankers on

allopatric species of *Populus* such as *P. trichocarpa* and exotic hybrids (Bier 1939; Strobl 1992; LeBoldus et al. 2010). Like many ascomycetes, *S. musiva* is a filamentous fungus and has a life cycle which is primarily asexual and haploid (Dhillon et al. 2019). Primary infection occurs in the spring at leaf emergence. Typically, ascospores released from pseudothecia, which overwinter in leaf debris at the soil surface, infect newly emerging leaves and shoots (Bier 1939; Feau et al. 2005a). Throughout the course of the growing season, pycnidia containing conidia produced on symptomatic leaf and stem tissue, serve as secondary inoculum (Waterman 1954; Feau et al. 2005a). Conidia penetrate through natural openings, lenticels, and directly into susceptible leaf and stem tissue (Qin and LeBoldus 2014; Abraham et al. 2019). After penetration, hyphae ramify in the intercellular spaces, leading to symptom development on leaves and woody tissue in as little as three weeks (Long et al. 1986; Weiland and Stanosz 2007; Qin and LeBoldus 2014; Abraham et al. 2019).

Leaf spot caused by *S. musiva* may lead to premature defoliation and reduced photosynthesis (Feau et al. 2010; Foster et al. 2015). Cankers on susceptible species may coalesce, girdling the stem and causing tree mortality (Bier 1939; Long et al. 1986; Spielman et al. 1986; Dunnell and LeBoldus 2016). In severe cases, biomass losses can be greater than 60% of total yield, and complete plantation failure may occur (McNabb et al. 1982; Newcombe and Ostry 2001). For example, in a mixed-hybrid plantation, 69% of the trees lost up to one-third of their height seven years after planting due to

breakage at cankers (Ostry et al. 1989). Stands of susceptible, pure *Populus* species are also at risk. In a genetics trial of *P. balsamifera* within its natural range, 56 genotypes were infected with at least one canker 7 years after planting (LeBoldus et al. 2009).

Although fungicides have been shown to reduce the number of cankers by as much as 2.5-fold, and biological control methods were able to reduce leaf spot severity rankings by approximately 1.0 on a scale of 0.0-8.0, the costs associated with numerous, repeated applications of chemical and biological agents makes these approaches impractical (Carbon 1972; Ostry and McNabb 1985; Ostry 1987; Gyenis et al. 2003; Feau et al. 2010). Similarly, cultural approaches such as burying leaves in the fall failed to reduce disease incidence (Ostry and McNabb 1983). As a result, planting genetically diverse, resistant genotypes is widely recommended for Septoria stem canker and leaf spot management (Ostry and McNabb 1985; Feau et al. 2010).

Sphaerulina musiva has been identified far from its native habitat in the northeastern United States and as far away as Brazil, Argentina, and regions of Asia (Sivanesan 1990; Maxwell et al. 1997; Santos et al. 2010). Anthropogenically-driven movement of infected poplar material is believed to play a large role in the development of infections outside of the native range of *S. musiva* (Sakalidis et al. 2016; Tabima et al. 2019). In 2007, *S. musiva* was identified in the Fraser Valley of British Columbia (BC) on hybrid poplar in commercial stool beds, within the native range of *P. trichocarpa*, a susceptible species (Callan et al. 2007; Richardson et al. 2014).

Due to the severity of *S. musiva* stem canker infections on commercially important hybrids and the susceptibility of *Populus* species native to the Pacific Northwest (PNW), including *P. trichocarpa*, identification of putative resistance genes will help breeding programs develop planting stock resistant to stem canker. General patterns of stem canker resistance have been observed on broad taxonomic levels, where species belonging to Section Aigeiros (including *P. deltoides* and *P. nigra*) are resistant to stem cankers and Section Tacahamaca (including *P. trichocarpa* and *P. balsamifera*) and its interspecific hybrids are susceptible to stem canker (Newcombe and Ostry 2001; Liang et al. 2014). Newcombe and Ostry (2001) obtained qualitative evidence that resistance to *S. musiva* stem canker is recessive based on a *P. trichocarpa* × *P. deltoides* (T × D) pseudo-backcross population (Family 342). However, in order to test their hypothesis, the authors suggested that inoculations needed to be conducted under controlled conditions (Newcombe and Ostry 2001).

To date, the hypothesis that resistance to Septoria stem canker is under recessive control has not been tested as suggested by Newcombe and Ostry (2001). As a result, an experiment was designed to: 1) evaluate the segregation of phenotypes for evidence in support of the hypothesis of recessive resistance; 2) detect associations (QTL) between the genotype of hybrid poplar and Septoria stem canker in a pseudo-backcross family; 3) determine if detected QTL are consistent with other reports on resistance of *Populus* to Septoria stem canker; and 4) identify candidate genes

encompassed by significant QTL using evidence of positive selection (Ka/Ks > 1) and gene annotations (Broman et al. 2003).

2.2. Materials and methods

2.2.1. QTL mapping population and plant propagation

A pseudo-backcross population (52-124) was developed by crossing a female *P. trichocarpa* from Washington State (93-968) and a male *P. deltoides* from southern Illinois (ILL-101; Muchero et al. 2015). This resulted in an F_1 hybrid female genotype (52-225), which was crossed with a second, different male *P. deltoides* from Minnesota (D124) due to *Populus* intolerance to inbreeding. A total of 700 F_2 progeny were produced. Using Illumina Infinium Bead Array technology, the 700 pseudo-backcross progeny were genotyped at 5,031 SNP loci. After filtering, the final genetic map contained 3,751 SNP markers spread across 19 linkage groups, 3,707 of which were used in this study (Muchero et al. 2015). In August 2017, 284 genotypes, a subset of the original 700, were planted at Oregon State University's Botany and Plant Pathology (BPP) Farm in Corvallis, OR (44°34'02.9"N 123°14'41.9"W). The three parents (93-968, 52-225, and D124) used in the development of the pseudo-backcross population were also included in the planting.

In January 2018, cuttings were collected from all 284 genotypes. A total of 10 dormant hardwood cuttings were collected from each individual. Ramets were all cut to

a length of 10 cm and planted in individual cone-tainers (Ray Leach SC10 Super Cone-tainers; Stuewe and Sons, Inc., Tangent, OR, U.S.) with pre-mixed soil (SunGro Professional Mix #8; SunGro Horticulture Ltd., Agawam, MA, U.S.) and 1 tablespoon of Osmocote slow release fertilizer (15-9-12; N-P-K; 7.0% NH3-N, 8.0% NO3-N, 9.0% P2O5, 12.0% K2O, 1.0% Mg, 2.3% S, 0.02% B, 0.05% Cu, 0.45% Fe, 0.23% chelated Fe, 0.06% Mn, 0.02% Mo, 0.05% Zn; Scotts Osmocote Plus; Scotts Company Ltd., Marysville, OH, U.S.). Greenhouse growth conditions were an 18-hour photoperiod supplemented by 600-watt high-pressure sodium lamps and a 20°C/16°C (day/night) temperature regime. Trees were fertilized bimonthly with an additional 20-20-20 (N-P-K) liquid fertilizer (Scotts Peters Professional; Scotts Company, Ltd., Marysville, OH, U.S.).

2.2.2. Fungal cultivation, inoculation, and phenotyping

Three isolates of *S. musiva* collected in West Virginia (WV-2, WV-3, WV-4) were chosen for inoculation and were isolated from infected trees in the 52-124 population planted at the West Virginia University agronomy farm in WV (39°39’32”N 79°54’19”W). Isolations were made by soaking cankers in a 5% sodium hypochlorite (NaClO) solution for 2 minutes, rinsing cankers twice with sterile distilled water, removing the bark from the stem, excising a 5-mm piece of wood from the margin of the necrotic tissue, and plating on a K-V8 media [180 mL V8 juice (Campbell Soup Company, Camden, NJ, U.S.), 2 g calcium carbonate, 20 g bacteriological agar, and 820 mL deionized water] amended

with Streptomycin sulphate (100 mg/L) and Chloramphenicol (246 mg/L). The plates were wrapped in parafilm, incubated at room temperature (ca. 20°C) under continuous light for seven days, and transferred to new K-V8 plates. Single spore isolates were made, and the species of the isolated fungus was confirmed using morphology and multilocus genotyping prior to storage in a 50% glycerol solution at -80°C (Sivanesan 1990; Stanosz and Stanosz 2002; Dunnell et al. 2016).

The three WV isolates were removed from cold storage and plated on K-V8 medium. Each isolate was plated on 8 plates, for a total of 24 plates. The cultures were incubated at room temperature in parafilm-sealed plates under full-spectrum fluorescent bulbs (Sylvania, Wilmington, MA, U.S.; Osram Gmbh, Munich, Germany) for one week. Fungal colonies were sub-cultured onto new K-V8 plates without antibiotics. Five 5-mm pieces of mycelium were placed on each of 75 plates, per isolate, for a total of 225 plates. The fungus grew for approximately three weeks at which point conidia from sporulating colonies were harvested. Briefly, conidia were dislodged by flooding the plates with 1 mL of deionized water, gently rubbing the surface of the colonies with a sterile inoculation loop and collecting the resulting spore suspension. The spore suspension from each flooded plate was combined to make a single spore suspension for each isolate. The concentration of each isolate's spore suspension was adjusted to 10^6 spores/mL using a hemocytometer. All three suspensions were then combined into a single bulk spore suspension which was used for inoculations.

The experimental design was a randomized complete block design with 6 blocks (5 experimental blocks and 1 non-inoculated control). Each of the 284 genotypes occurred once per block. Inoculations were conducted once the trees were approximately 30 cm tall. Initially, trees were moved from the greenhouse and their heights were measured. Each rack of 20 trees was placed in a black plastic bag, the trees were sprayed with the bulk spore suspension until runoff (~15mL/tree), and the bags were sealed for 48 hours at room temperature. After incubation, the racks were returned to their original placement in the greenhouse and the plastic bags were removed. Three weeks after inoculation, the number of cankers on the primary stem was counted, and disease severity was scored on a 1 to 5 scale (Dunnell et al. 2016). In this scale, 1 indicates no disease and 5 indicates severe stem cankers and girdling (Dunnell et al. 2016). All counting and scoring for all blocks were performed in a single day.

2.2.3. Genotyping and QTL mapping

The genetic map used in this study contained SNP genotyping data for 230 of the 284 genotypes for which canker counts and disease severity scores were recorded in this study. The phenotyping data was merged with the genetic map using the merge() function from the R/base package (R Core Team 2017). Both the total dataset and the subset dataset were tested for quantitative evidence supporting the recessive resistance

hypothesis. The data used for modelling and QTL mapping consisted of genotyping and phenotyping data from 230 of the original 284 genotypes.

2.2.4. Population structure analysis and Chi-squared phenotype segregation test

Population number (K) and genotype assignment was conducted using STRUCTURE software (Pritchard et al. 2000; Hubisz et al. 2009). The analysis was performed on the reduced set of 230 genotypes for K = 1-10, with 15 runs at each K, a burn-in length of 10,000 and a run length of 20,000. The ideal K was determined using CLUMPAK cluster mode identification (Evanno et al. 2005; Kopelman et al. 2015). Population structure was accounted for in constructed models as a fixed effect by assigning each genotype to one of the two clusters based on the posterior probabilities estimated by STRUCTURE.

Newcombe and Ostry (2001) hypothesized that resistance to stem canker was recessive and should segregate in a 1:1 ratio in the pseudo-backcross F_2 progeny of the 52-124 population. Deviation from the expected 1:1 ratio of resistant (canker count = 0; disease severity = 1) and susceptible (canker count > 0; disease severity > 1) genotypes was tested using a Chi-Squared Goodness-of-Fit Test with a Yates correction ($\alpha = 0.05$). The test was conducted twice for both canker count data and disease severity data, once on the set of data including individuals from all 284 genotypes inoculated, and again on the subset of data including only individuals from the 230 genotypes for which marker data was available (Tables 2.1-2.4).

2.2.5. Canker count model

Data was analyzed using R studio (R Core Team 2017). For all analyses described below, significance was assessed at α = 0.05. A generalized linear mixed model (GLMM) with a negative binomial distribution was fit to the canker count data with the R/lme4 package (Bates et al. 2015). The fixed effects were height at the time of inoculation and cluster. The random effects included genotype, block, and rack nested within block (rack(block)). The model assumptions were evaluated by one-sample Kolmogorov-Smirnov test (p = 0.0632), a non-parametric dispersion test (p = 0.049), and a test for zero-inflation (p = 0.441) using the R/DHARMa package with 10,000 permutations (Hartig, 2016). In addition, the scaled Pearson residuals were evaluated for patterns.

The model used to analyse the data was:

$$\log(\lambda_t) = \beta_0 + \beta_1 I.cluster_t + \beta_2 height_t + (b_i)_t + (c_i)_t + (d_r)_t$$

where:

$I.cluster_t$ is 1 if the t^{th} tree was assigned to Cluster 1 and 0 otherwise;

$height_t$ is the value of the continuous explanatory variable height (to nearest 1/10 cm) for the t^{th} tree;

β_0 is the log mean canker count for tree in Cluster 2 when height is 0;

β_1 is the difference in the log mean canker count between trees assigned to Cluster 1 compared to Cluster 2 when height is 0;

β_2 is the change in log mean canker count per 1-cm increase in height;

b_i is the random effect of the i^{th} block on log mean canker count;

$b_i \sim N(0, \sigma_{block}^2)$ and b_i and b_i' are independent;

c_j is the random effect of the j^{th} rack on log mean canker count;

$c_j \sim N(0, \sigma_{rack}^2)$ and c_j and c_j' are independent;

d_r is the random effect of the r^{th} genotype on log mean canker count;

$d_r \sim N(0, \sigma_{genotype}^2)$ and d_r and d_r' are independent.

The significance of fixed effects was estimated by the R/lmerTest package using Satterthwaite's method (Satterthwaite 1964; Stroup 2014; Kuznetsova et al. 2017). The significance of random effects was estimated using a Chi-squared likelihood ratio test with the anova() function from the lme4 package in R; the contribution of each random effect to the total variance explained by random effects was determined as a percentage (see Table 2.5; Bates et al. 2015). The best linear unbiased predictors (BLUPs) were extracted from the random effects for each genotype (Bates et al. 2015). The BLUPs were used to estimate QTL for disease resistance with the composite interval mapping function in the R/qtl package with 1000 permutations (Broman et al. 2003). The closest marker to the QTL was extracted using the find.marker() function.

2.2.6. Marker-effect model

To obtain an estimate of how the alleles at QTL peaks affect the multiplicative difference in the mean canker count, the allele state ("A" or "H") from the genetic map at the appropriate marker was added to the model as a new fixed effect. The "A" describes the fixed, homozygous allele state of the *P. deltoides* parents and "H" describes the heterozygous allele state expected from a cross between fixed homozygous dominant and fixed homozygous recessive parents. Significance testing of the fixed and random effects, and analysis of model assumptions, were performed as described above (Bates et al. 2015; Hartig 2016). Comparisons between the mean canker count of individuals with an "A" or "H" allele state were made using the R/emmeans package (Length and Herve 2019). The 95% confidence intervals (CI) were calculated by profiling the likelihood with the confint() function from the lme4 package in R (Bates et al. 2015). Heritability for significant QTL was estimated at the scale of the model (latent scale) and the scale of the data (observation scale) after accounting for fixed effects, using the R/QGglmm package (de Villemereuil et al. 2016).

2.2.7. Disease severity model

In order to estimate disease severity BLUPs and generate a QTL map, a linear mixed model (LMM) with the fixed parameters of height and population structure, as well as the random effects of genotype, block, and rack(block) was fit to the disease severity data. Model selection, fit, BLUP estimation, QTL map generation, and allele effect

estimation were conducted in an identical manner to that described above. Heritability for significant disease severity QTL was estimated at the response scale by dividing the genotypic variance component by the total phenotypic variance ($H^2 = [\delta_g^2/(\delta_g^2 + \delta_e^2)]$) where δ_g^2 is the genotypic variance component and δ_e^2 is the residual variance.

2.2.8. Candidate gene selection

In order to identify potential candidate genes at significant QTL, all genes within a 1,000-Kb window centered on the QTL peak were extracted from the *P. trichocarpa* and *P. deltoides* reference genomes (Supplementary Table 2.1; Goodstein et al. 2012; Phytozome v3.0). Gene orthology between *Populus* species for gene intervals was determined using MCScanX (Wang et al. 2012). BLASTP was used to compare predicted proteins between the *P. trichocarpa* and *P. deltoides* intervals, and MCScanX was used to stitch together collinear segments (Altschul et al. 2005). Genes were considered orthologous when pairs existed in collinear order based on BLASTP E-score < 1E-180, the two genes matched with > 89% identity, and the non-synonymous mutation rate (Ka) < 0.2. Gene annotations, domain compositions, and gene ontology (GO) terms were summarized for each gene.

The ratio of non-synonymous to synonymous mutation rate (Ka/Ks) was calculated for all putatively orthologous genes collected above. Values of (Ka/Ks) > 1 were considered indications of positive selection and may suggest that mutations were

kept due to selective advantage. Potential candidate genes were selected for further discussion using the following criteria: the 1,000 kb window, gene annotations, and (Ka/Ks) > 1 (Fay et al. 2001; Xing and Lee 2005; Zhang et al. 2006). All amino acid sequence alignments were performed using NCBI BLASTP with default settings (Altschul et al. 2005).

2.3. Results

2.3.1. Phenotyping and population structure

Canker number ranged from 0-78 (Fig. 2.1, Table 2.1). Disease severity scores ranged from 1-5 (Fig. 2.2, Table 2.2). All control plants were free of cankers and were excluded from further data analysis. The ideal number of subpopulations (K), determined by STRUCTURE analysis, was K = 2 (Fig. 2.3; Evanno et al. 2005; Kopelman et al. 2015). A total of 104 genotypes were assigned to Cluster 1, and 126 genotypes were assigned to Cluster 2.

The Yates-corrected Chi-Squared Goodness-of-Fit Test conducted on the canker count phenotypes, indicated a deviation from the expected 1:1 ratio of resistant and susceptible genotypes in the F_2 progeny, before ($p < 0.0001$, $df = 1$) and after ($p < 0.0001$, $df = 1$) filtering (Table 2.3). The same test performed for the disease severity phenotype indicated that there was no evidence of deviation from the expected 1:1

ratio of resistant and susceptible genotypes in the F_2 progeny before ($p = 0.417$; $df = 1$) or after ($p = 0.952$; $df = 1$) filtering (Table 2.4).

2.3.2. Canker count model and QTL map

The negative binomial GLMM used to estimate BLUPs included the fixed effects of height ($p < 0.0001$) and population cluster ($p < 0.0001$) as well as the random effects of genotype ($p < 0.0001$), block ($p < 0.0001$), and rack(block) ($p < 0.0001$). The genotype effect explained 42.25% of the variance, the block effect explained 35.43% of the variance, and the rack(block) effect explained the remaining 22.32% of the variance (Table 2.5). The one-sample Kolmogorov-Smirnov test ($p = 0.0632$), non-parametric dispersion test ($p = 0.049$), test for zero-inflation ($p = 0.441$), and display of standardized residuals versus predicted values were consistent with the assumptions for a negative binomial GLMM. Using the BLUPs generated by this model, a single significant QTL was detected (marker position = 75 cM; $p = 0.021$). This marker (c16.loc75) is a pseudomarker with its position determined by interpolation between two real markers. This pseudomarker was located at 75cM on Linkage Group 16 and was positioned between two real markers located at 74.56263779 cM (scaffold_16_12428393) and 77.46884676 cM (scaffold_16_12536991; Fig. 2.4; Fig. 2.5).

2.3.3. Marker-effect model

After adding a fixed effect to account for the allele state of individuals at the significant QTL on Linkage Group 16 (scaffold_16_12428393), the model included fixed effects for height at the time of inoculation ($p < 0.0001$), population cluster ($p = 0.0124$), and allele state ($p < 0.0001$) as well as the random effects of genotype ($p < 0.0001$), block ($p < 0.0001$), and rack(block) ($p < 0.0001$). The genotype effect in this model explained 36.86% of the variance, the block effect explained 38.26%, and the rack(block) effect explained 24.88% of the variance (Table 2.6). The variability of the genotype effect was smaller when the marker effect was added to the model (Tables 2.5-2.6). The one-sample Kolmogorov-Smirnov test ($p = 0.1046$), a display of standardized residuals versus predicted values, a non-parametric dispersion test ($p = 0.0398$), and a test for zero-inflation ($p = 0.4538$) were consistent with the assumptions for a negative binomial GLMM. The fixed effect for allele state indicated that the mean number of observed cankers was 1.86-fold higher for individuals with the "H" allele state compared to those with the "A" allele state (95% Confidence Intervals: 1.44X to 2.37X).

In addition to the significant QTL on Linkage Group 16, a second QTL on Linkage Group 8 was observed (marker = scaffold_8_1726500; marker position = 17.202838; $p = 0.087$). This QTL did not pass the significance threshold, and the mean number of observed cankers was estimated to be approximately 1.62-fold higher for individuals with the "A" allele state compared to those with the "H" allele state (95% Confidence interval: 1.25X to 2.04X).

Heritability of the canker count phenotype was estimated at the model-scale (H^2 = 0.4225) and the observational scale (H^2 = 0.0261). The heritability estimates were similar for the model which included the fixed effect for allele state, with the latent scale heritability (H^2 = 0.3686) and observation-scale heritability (H^2 = 0.0229) slightly lower than the reduced model.

2.3.4. Disease severity model and QTL map

The linear mixed model for disease severity had fixed effects for height ($p < 0.0001$) and population assignment ($p = 0.0060$), and random effects for genotype ($p < 0.0001$), block ($p < 0.0043$), and rack(block) ($p < 0.0001$). Two peaks were observed on the resulting QTL map (Fig. 2.6). The first QTL was identical to that identified for canker count (marker = scaffold_16_12428393; marker position = 74.56263779 cM; $p < 0.0001$). The fixed effect for allele state indicated that the mean disease severity was 1.25-fold higher for individuals with the “H” allele state compared to those with the “A” allele state (95% Confidence Intervals: 1.10X to 1.41X).

The second QTL (marker = scaffold_624_918; marker position = 137.7449484; $p = 0.06$) was located on chromosome 2 (Fig. 2.6). The fixed effect for allele state for this marker indicated that the mean disease severity was 1.18-fold higher for individuals with the “A” allele state at scaffold_624_918 compared to individuals with the “H” allele state (95% Confidence Intervals: 1.05X to 1.31X).

Heritability of the disease severity phenotype at the response scale was estimated. The heritability estimates were similar for the reduced model ($H^2 = 0.152$) and the model which included the fixed effect for allele state ($H^2 = 0.126$), although accounting for the allele state reduced heritability.

2.3.5. Candidate gene selection

The 1,000-kilobase window centered on marker scaffold_16_12428393 contained a total of 151 genes, 114 of these genes were orthologous between *P. trichocarpa* and *P. deltoides* (Supplementary Table 2.1), 23 were in the *P. deltoides* genome and lacked an identified *P. trichocarpa* orthologue (Supplementary Table 2.2), and 14 genes were in the *P. trichocarpa* genome and lacked an identified *P. deltoides* orthologue (Supplementary Table 2.3). Of the 114 detected orthologous genes, 15 had (Ka/Ks) > 1 (Supplementary Table 2.4). Gene annotations related to plant immune response were used to further reduce the list of candidate genes (Table 2.9). A Concanavalin A-like lectin protein kinase family protein (Potri.016G122700/ Podel.16G128900) was selected for further discussion based upon a high (Ka/Ks) ~ 3.14 and the well-documented history of lectin protein kinase function in plant immunity (Bouwmeester and Govers 2009; Joshi et al. 2010; Huang et al. 2013; Singh and Zimmerli 2013). Although a hydroxyproline-rich glycoprotein family protein was found to have a (Ka/Ks) ~ 1.12, previous studies have found evidence of a relationship with plant wounding and fungal

infection (Showalter et al. 1985; Corbin et al. 1987). Two other orthologous genes had (Ka/Ks) > 2.0 but had limited available gene annotation or published literature (*P. trichocarpa* gene models: Potri.016G121600 and Potri.016G125800).

2.4. Discussion

Newcombe and Ostry (2001) presented a hypothesis that stem canker resistance in an F_2 pseudo-backcross with a TD × D lineage (such as the 52-124 family) is under the control of a single recessive gene, although single-gene control could not be confirmed. Under this hypothesis, the 52-124 family is expected to segregate in a 1:1 ratio of Resistant to Susceptible progeny, assuming the genes are segregating in a Mendelian fashion. The data from the greenhouse QTL study described here supports this hypothesis. Although the corresponding Yates-corrected Chi-Square analysis for the canker count phenotype showed strong evidence ($p < 0.0001$) that the ratio of resistant to susceptible individuals within the population was not 1:1 as expected (Table 2.3), the segregation of disease severity scores did not deviate from expectation.

A single canker can vary drastically between host genotypes and between individuals. It is possible that canker count phenotyping alone cannot provide a complete perspective of resistance or susceptibility, thereby affecting the results of the Chi-Squared analyses. The heritability of the full data set and the subset used for association mapping of the canker count phenotype was $H^2 = 0.4225$ and $H^2 = 0.3686$,

respectively. These data indicate a moderately-high percentage of the total phenotypic variance was due to genetic factors. On the other hand, the heritability of the full data and the subset calculated from the disease severity model was $H^2 = 0.152$ and $H^2 = 0.126$, respectively, indicating a weak genetic influence on total phenotypic variance. The low observation scale heritability results for the negative binomial GLMM for both the full data set and the subset used for association mapping reveal the inherent variability involved when using a negative binomial distribution rather than a normal distribution modelling approach. Interestingly, the heritability for Septoria leaf spot for the 52-124 field data reported by Simon *et al.* (unpublished) was $H^2 = 0.227$, although the heritability of the Septoria stem canker phenotype was not reported.

All models described contained a significant rack(block) effect. This significance is likely due to the haphazard assignment of individuals to racks and blocks, and future studies may be able to minimize block and rack by using a truly randomized assignment process. Despite the significant amount of total variation explained by rack(block) and block alone in the reduced negative binomial GLMM, the genotype effect still explained the largest amount of variation (Table 2.5). After adding an additional fixed effect related to the allele state at marker scaffold_16_12428393 on Linkage Group 16, the genotype described 36.86% of the total variation described by random effects, although the block effect explained the most variance in this model (Table 2.6). Out of the random effects in the disease severity LMM for both the reduced and full models,

genotype explained the largest proportion of the explained variance (13.90% and 11.44%, respectively; Tables 2.7-2.8).

It should be noted that the R/DHARMa package detected that the disease severity model may not be ideal for the categorical, rating response type (One-Sample Kolmogorov-Smirnov test p-value = 5.24E-10). However, the use of linear mixed models to fit categorical disease rating data is well-documented within the field, the plot of scaled Pearson residuals did not indicate any obvious patterns, and the peak on Linkage Group 16 was consistent between phenotypes and models (LeBoldus et al. 2007; Dunnell 2016; Dunnell and LeBoldus 2016; Dunnell et al. 2016). The disease severity LMM for both the full data set and the filtered subset indicated high amounts of residual variance, providing further evidence that more variance may be explained with a better-fitting model and truly randomized block and rack assignment. Additionally, the individuals used for this greenhouse QTL study comprised 284 genotypes (230 after filtering for marker data)— a subset of a larger collection of 52-124 progeny. It is possible that the subset of individuals used in this experiment did not fully capture the genetic and phenotypic variation of the entire family.

The genetic map comprised single nucleotide polymorphism (SNP) markers determined to be fixed for either *P. deltoides* or *P. trichocarpa* spread across the 19 linkage groups. The allele state “A” was indicative of only *P. deltoides* contribution at a specific marker, whereas the allele state “H” was indicative of a hybridization between

the *P. deltoides* and *P. trichocarpa*. As such, the effect of maternal, *P. trichocarpa* contributions could be observed at markers with allele state “H” compared to those with allele state “A”. Given the hypothesis outlined by Newcome and Ostry (2001) of recessive control of resistance to Septoria stem canker, this “H” allele state would be sufficient to confer susceptibility to stem canker in *Populus* hybrids. Thus, genes which may be controlling disease susceptibility or resistance were expected to have a larger canker count for individuals with the “H” allele state as compared to the “A” allele state.

The field study conducted by Simon *et al.* (unpublished) at the WVU Agronomy farm on a larger population of 52-124 progeny rated stem canker phenotypes on a scale of 0-5 and used the BLUPs estimated by a binary model to localize QTL. These data also found a significant QTL on Linkage Group 16, with the strongest association being at marker scaffold_16_8973683, located at 60.81 cM and 9,704,065 base pairs (Fig. 2.5). The QTL identified in the field study and this greenhouse study occur at different positions, separated by approximately 13.75 cM and 3,000,000 bp. However, interpretation of the difference between location of the QTL from the greenhouse data reported here and the QTL from the field data reported by Simon *et al.* (unpublished) may be difficult to interpret. The position of the QTL may have been affected by the number of progeny tested in the greenhouse study, the difference in phenotyping, or by the fact that the field study was conducted on mature trees in the late stages of damage caused by different diseases and pests. However, the localization of a QTL to the same

linkage group likely indicates the presence of an important disease-regulating gene somewhere near both detected QTL.

One additional peak was produced each by the canker count negative binomial GLMM and the disease severity LMM, although they did not pass the $\alpha = 0.05$ significance threshold set for this greenhouse study. The negative binomial model localized an additional QTL ($p = 0.087$) to marker scaffold_8_1726500 at 17.20 cM on Linkage Group 8. However, the mean number of observed cankers was estimated to be 1.62-fold higher for individuals with allele state “A” compared to individuals with allele state “H”. It was expected that significant markers related to Septoria stem canker would be associated with fewer cankers and lower disease severity in individuals with allele state “A” compared to individuals with allele state “H”. The QTL on Linkage Group 8 shows evidence of the opposite relationship. The additional QTL ($p = 0.06$) localized to marker scaffold_624_918 at 137.75 cM on Linkage Group 2 by the disease severity LMM behaved similarly. The mean disease severity for individuals with allele state “A” at this marker was estimated to be 1.18-fold higher compared to the mean disease severity for individuals with the hybrid-like allele state “H”. It is possible that these two QTL may have unrealized impacts on resistance which are masked by the greater effect of the QTL on Linkage Group 16. An alternative possibility is that the two additional QTL were model artefacts from the BLUP estimation or other associations due to chance.

Some QTL studies advocate for the "LOD drop-off method" to determine the size of the window from which candidate genes are selected. In QTL studies, the strength of association between a marker and a phenotype is measured by the LOD (logarithm of the odds) score and a larger LOD score indicates a stronger statistical association between the marker and the trait (Young 1996; Broman et al. 2003; Collard et al. 2004). In the LOD drop-off method, also known as the LOD confidence interval method, candidate genes are collected from a window centered on the QTL with boundaries defined by a certain loss of LOD, usually 1 LOD (Pflieger et al. 1999; Kumar et al. 2006). Using the LOD drop-off method, windows centered on the QTL presented here varied immensely, likely due to variable marker coverage in the linkage map (Fig. 2.7) or inaccuracies in genotyping and phenotyping. For example, the window centered on the significant QTL on Linkage Group 16 using the LOD drop-off method was 0.761 Mb and 0.719 Mb for the canker count and the disease severity QTL maps, respectively. However, the window centered on the QTL on Linkage Group 2 on the disease severity QTL map was 16.353 Mb and the window for the QTL on Linkage Group 8 on the canker count QTL map was only 1.387 Mb. Thus, a fixed, 1.0 Mb window size was considered a more conservative approach and is consistent with the methodology used in the past on this 52-124 family (Simon et al., unpublished).

A total of 151 gene models were collected from Phytozome as described and consisted of orthologous genes as well as genes unique to either *P. trichocarpa* or *P.*

deltoides. The *P. trichocarpa* gene models included two receptor-like proteins (RLP6 and RLP33) lacking a syntenic orthologue in *P. deltoides*. Similarly, the *P. deltoides* genome contained four receptor-like proteins (RLP6, RLP7, RLP19, and RLP33) lacking a syntenic orthologue in *P. trichocarpa*. The amino acid sequences of the gene models of RLP6 (Potri.016G127000 and Podel.16G132500) which were not determined to be orthologues were aligned with a BLASTP E-value of 0.0 and approximately 59.37% identity match with 99% query cover. Similarly, an alignment of the amino acid sequences of the non-orthologous RLP33 gene models (Potri.016G127100 and Podel.16G125900) produced a BLASTP E-value of 8E-47 and approximately 53.80% identity match, with 83% query cover. Due to the complicated evolutionary history of whole-genome duplication, chromosomal rearrangement, and tandem duplication events in *Populus*, it is possible that these genes are closely related, although not determined to be orthologues by the methods used here, which required Ka < 0.2, identity match > 80%, and synteny (Tuskan et al. 2006; Simon et al., unpublished). It is possible that the functional differences between these proteins may affect resistant or susceptible responses to Septoria stem canker and genes without orthologues may still be important to hybrid progeny. Additionally, the two receptor-like proteins unique to *P. deltoides* (RLP7 and RLP19) may explain a differential response to Septoria stem canker between *P. deltoides* and *P. trichocarpa.* The candidate genes reported by the GWAS leveraging SNP data from a population of pure *P. trichocarpa* included two

receptor-like proteins (RLP1 and RLP2) which were upregulated in resistant individuals 24 hours-post-inoculation, indicating that recognition of *S. musiva* may play an important role in conferring a resistant response in *Populus* (Muchero et al. 2018).

One of the most notable pairs of candidate genes identified were the orthologues of *P. trichocarpa* gene Potri.016G122700 and *P. deltoides* gene Podel.16G128900 (Table 2.9). This gene was predicted to have *Concanavalin A-like Lectin Protein Kinase family protein* or *Lectin Receptor Kinase A4.1* activity for *P. trichocarpa* and *P. deltoides*, respectively. The *Concanavalin-A like lectin protein kinase* gene had a high ratio of non-synonymous vs. synonymous mutations (Ka/Ks = 3.14) indicating potential positive selection between the orthologues. Evidence of positive selection may indicate that mutations in genes related to disease resistance (e.g. pathogen recognition) may have provided a selective advantage.

Lectin receptors, including lectin receptor kinases and lectin protein kinases, contribute to the plant innate immune system by recognizing microbial patterns, including carbohydrates present on the pathogen's cell surface (Singh and Zimmerli 2013; Vaid et al. 2013; Yang et al. 2016). Lectin protein kinases have extracellular lectin domains responsible for pattern recognition, and transmembrane and intracellular domains that activate signaling cascades that modulate downstream transcription and gene expression (Bouwmeester and Govers 2009; Joshi et al. 2010). Lectin receptors may also recognize damage-associated molecular patterns (DAMPs), as the Arabidopsis

lectin receptor kinase DORN1 was found to bind to extracellular ATP (Choi 2014; Tanaka et al. 2014). However, there is evidence that lectin receptor kinases can also be activated by abiotic stress such as high salinity (Joshi et al. 2010; Ye et al. 2017).

The recognition domain of Potri.016G122700 and Podel.16G128900 may be structured similarly to Concanavalin A (ConA), which is a lectin first purified from jack bean (Olson and Liener 1967). The orthologous candidate genes Potri.016G122700 and Podel.16G128900 were aligned to the amino acid sequence of jack bean Concanavalin A (GenBank accession P81461) with BLASTP and Potri.016G122700 had a 25.64% identity match over 47% of the amino acid sequence of jack bean ConA with an E-score of 2E-8. The *P. deltoides* orthologue, Podel.16G128900, covered approximately 97% of the amino acid sequence of ConA, and had a 29.17% identity match and an E-score of 1E-12.

ConA binds to cell-surface mannans on yeast and lyse the zoospores of several organisms at micromolar concentrations, including *Phytophthora cinnamomi* and *Phytophthora palmivora* (Sing and Bartnicki-Garcia 1975; Byrt et al. 1982; Goldstein and Poretz 2012). A study of the rice blast ascomycete pathogen *Magnaporthe grisea* found that a mucilage released at the apex of *M. grisea* conidia was necessary for surface adhesion, that ConA readily bound to a component of the mucilage, and that ConA could disrupt the adhesion of *M. grisea* conidia to surfaces (Hamer et al. 1988). Similar studies on the adhesion characteristics of *S. musiva* may provide experimental evidence supporting the role of Potri.016G122700 in conferring resistance. However, the

dissimilarities between ConA and the putative lectin receptor proteins in *P. trichocarpa* and *P. deltoides* likely indicate functional differences as well. A few other pairs of orthologous genes are worth mentioning due to relevant gene annotations or high (Ka/Ks).

Potri.016G129400 and Podel.16G135200 had a gene annotation of *hydroxyproline-rich glycoprotein family proteins* (Table 2.9). Hydroxyproline-rich glycoproteins (HPRGs) are an abundant family of structural proteins present in plant cell walls with a protein backbone with many hydroxyproline residues (Showalter et al. 1985; Sommer-Knudsen et al. 1998; Hijazi et al. 2014). HPRGs are thought to be cross-linked with polysaccharides during cell wall development, but this process is not well understood, and evidence of molecular interactions involving these proteins is sparse (Hijazi et al. 2014). Showalter *et al.* (1985) found that mRNA coding for HRPGs were upregulated in bean (*Phaseolus vulgaris*) in response to both artificial elicitor treatment and with specific interactions between bean cultivars and races of *Colletotrichum lindemuthianum*. In resistant cultivars undergoing a hypersensitive response, levels of mRNA of HRPGs were between 10 and 20-fold higher than seen in controls (Showalter et al. 1985). Similarly, Corbin *et al.* (1987) revealed qualitative evidence that several transcripts related to hydroxyproline-rich glycoproteins were differentially expressed when bean cells were wounded compared to when they were infected with the *Colletotrichum lindemuthianum* pathogen. However, HRPGs have also been associated

with many other functional roles in plant organisms, including responses to abiotic stress (Hijazi et al. 2014). Furthermore, there was little evidence of positive selection based upon a (Ka/Ks) ~ 1.12.

Potri.016G125800 and Podel.16G131100 had a (Ka/Ks) ~ 2.31 and the *P. deltoides* gene model was annotated as a BRCA2-like B protein whereas the *P. trichocarpa* gene model lacked an annotation. *BRCA2* is a very well-studied gene in mammalian systems due to implications related to cancer development (Suzuki et al. 1997; Jazaeri et a. 2002; van Beers et al. 2005; Joosse et al. 2012). The normal function of *BRCA2* in mammals is related to tumor suppression and DNA repair and recombination, but polymorphisms at this gene have been associated with a predisposition to cancer development (Patel et al. 1998; Scully and Livingston 2000; Venkitaraman 2001; Yoshida and Miki 2004; Couch et al. 2007). However, plant BRCA2 gene analogues have been found to be involved in the expression of plant defense genes and the production of DNA repair proteins (Wang et al. 2010; Song et al. 2011). *Arabidopsis thaliana brca2* mutants were found to be hypersusceptible ($p < 0.01$) to the bacterial pathogen *Pseudomonas syringae* pv. *maculicola* compared to the Col-0 control (Wang et al. 2010). Recent literature suggests that plant pathogens, including fungi and oomycetes as well as bacteria, frequently cause double-stranded breaks in the DNA of host cells and that BRCA2 plays a role in reducing the resulting damage (Song et al. 2011; Song and Bent 2014). Evidence has shown that double-stranded break repair can

be triggered by treatment with salicylic acid—a key component of plant response to biotrophic pathogens and an instigator of the hypersensitive response (Xu et al. 1994; Alvarez 2000; Shah 2003; Song and Bent 2014). An amino acid sequence alignment of the *P. deltoides* gene model to the *P. trichocarpa* gene model was performed and resulted in an identity score of 90.91% over 61% of the *P. trichocarpa* sequence. Differences in the functional capacity of BRCA2 proteins in *P. trichocarpa* and *P. deltoides* may, therefore, be related to an inability to repair double-stranded breaks, even when salicylic acid-associated defense pathways are activated.

This QTL study provides evidence that resistance to Septoria stem canker is recessive. Additionally, a single QTL strongly associated with stem canker resistance was localized to Linkage Group 16. Although the QTL identified by Simon *et al.* (unpublished) was approximately 3 Mb away from the QTL identified in this study, both were on Linkage Group 16. Furthermore, several promising candidate genes are encoded within the 1-Mb window centered on this QTL and may reveal more information upon characterization.

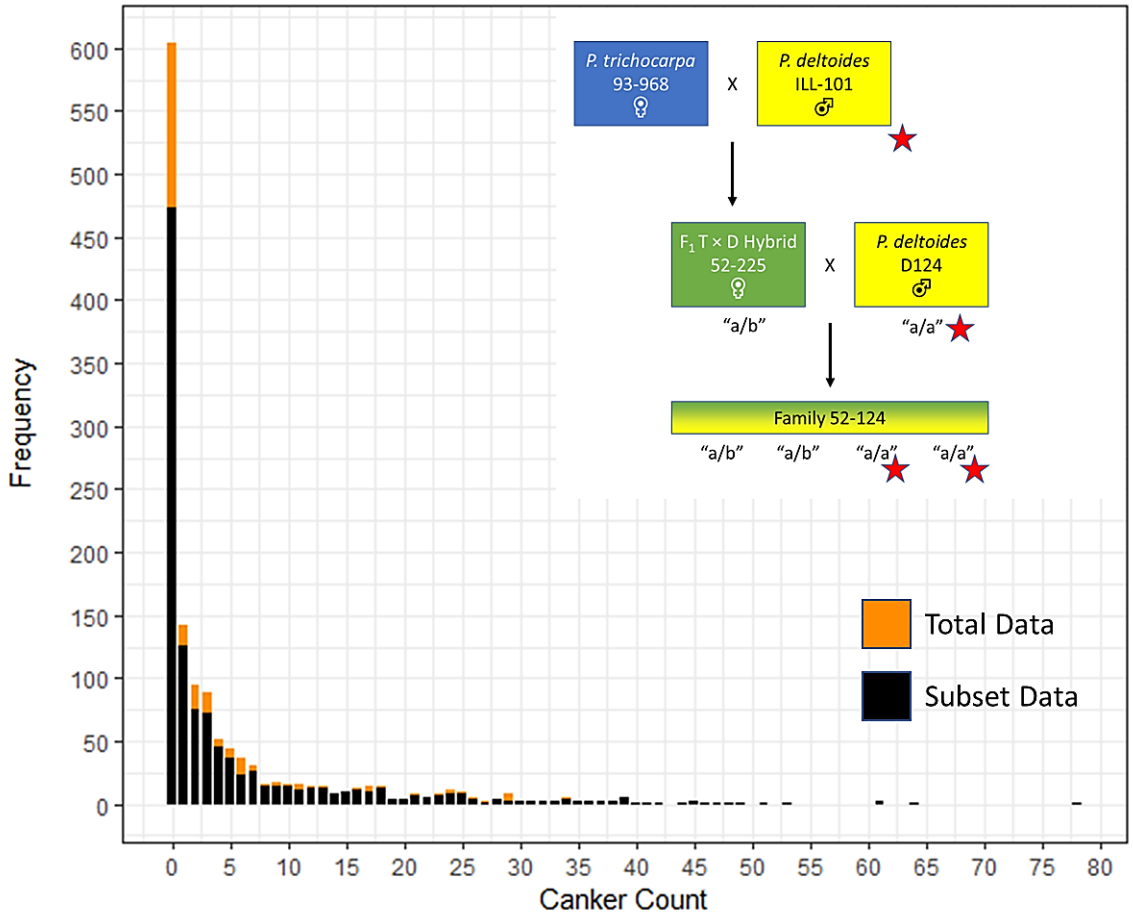

Fig. 2.1. Canker count data from non-control individuals. The subset of data used for QTL detection (black) are nested within the total data (orange). Inset: hybrid population background and associated marker types, where alleles with the red star indicate putatively resistant genotypes.

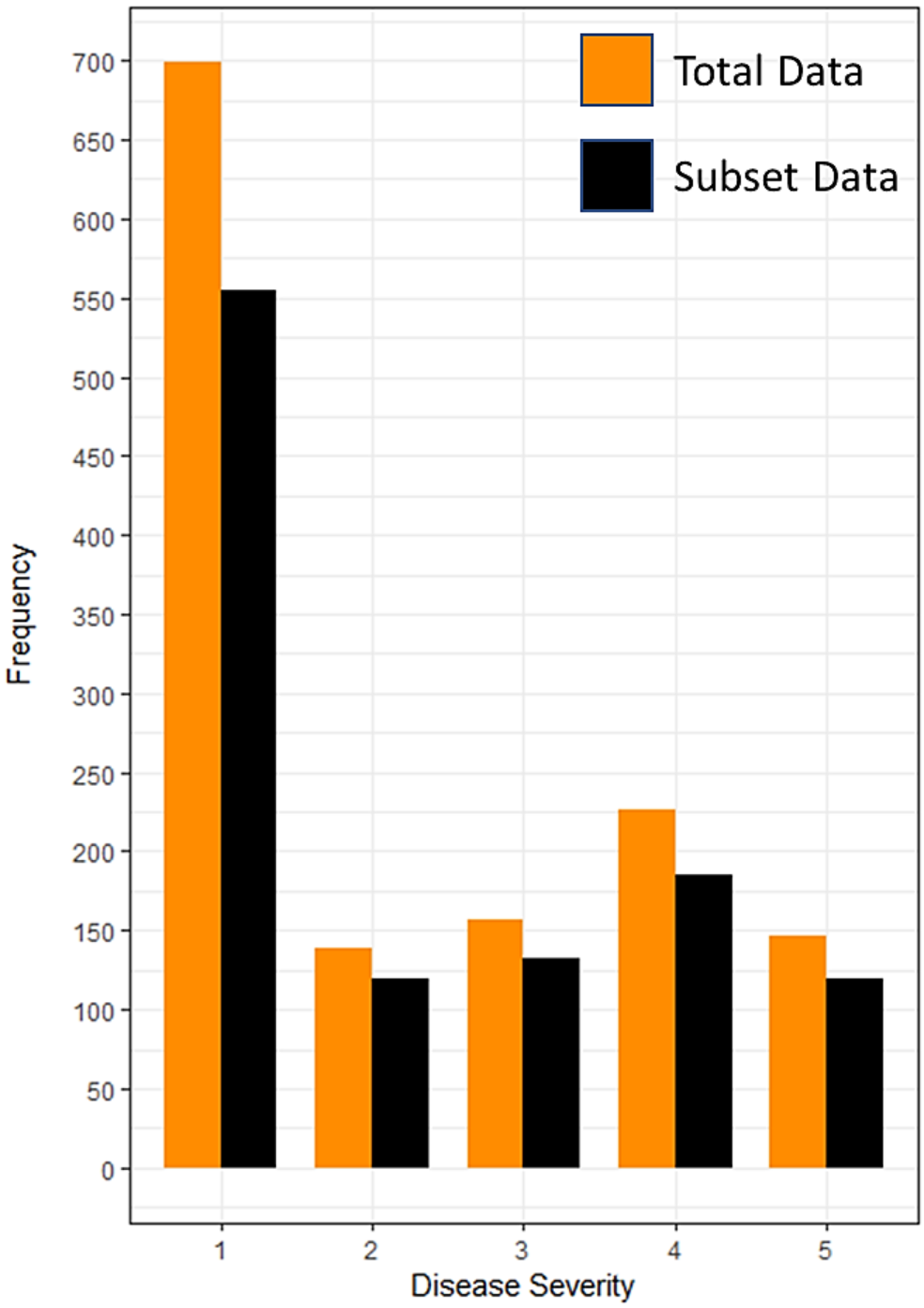

Fig. 2.2. Disease severities from non-control individuals. The subset of data used for QTL map construction are shown in black, and the total data is shown in orange.

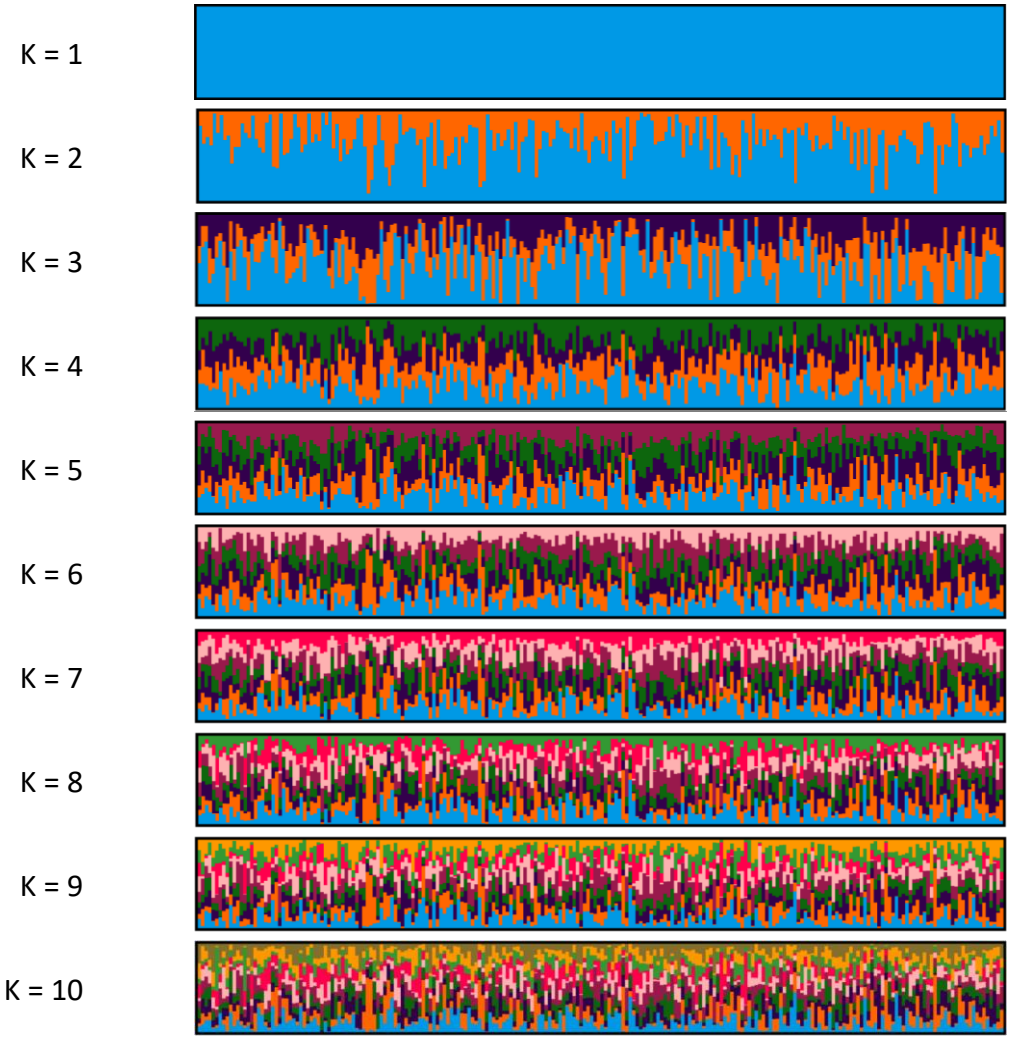

Fig. 2.3. Average STRUCTURE bar plots for the optimal number of subpopulations (clusters) for the 52-124 TD × D hybrid poplar family over 15 runs for K = 1-10. The ideal number of subpopulations was determined to be K = 2 by CLUMPAK cluster mode identification (Evanno et al. 2005; Kopelman et al. 2015).

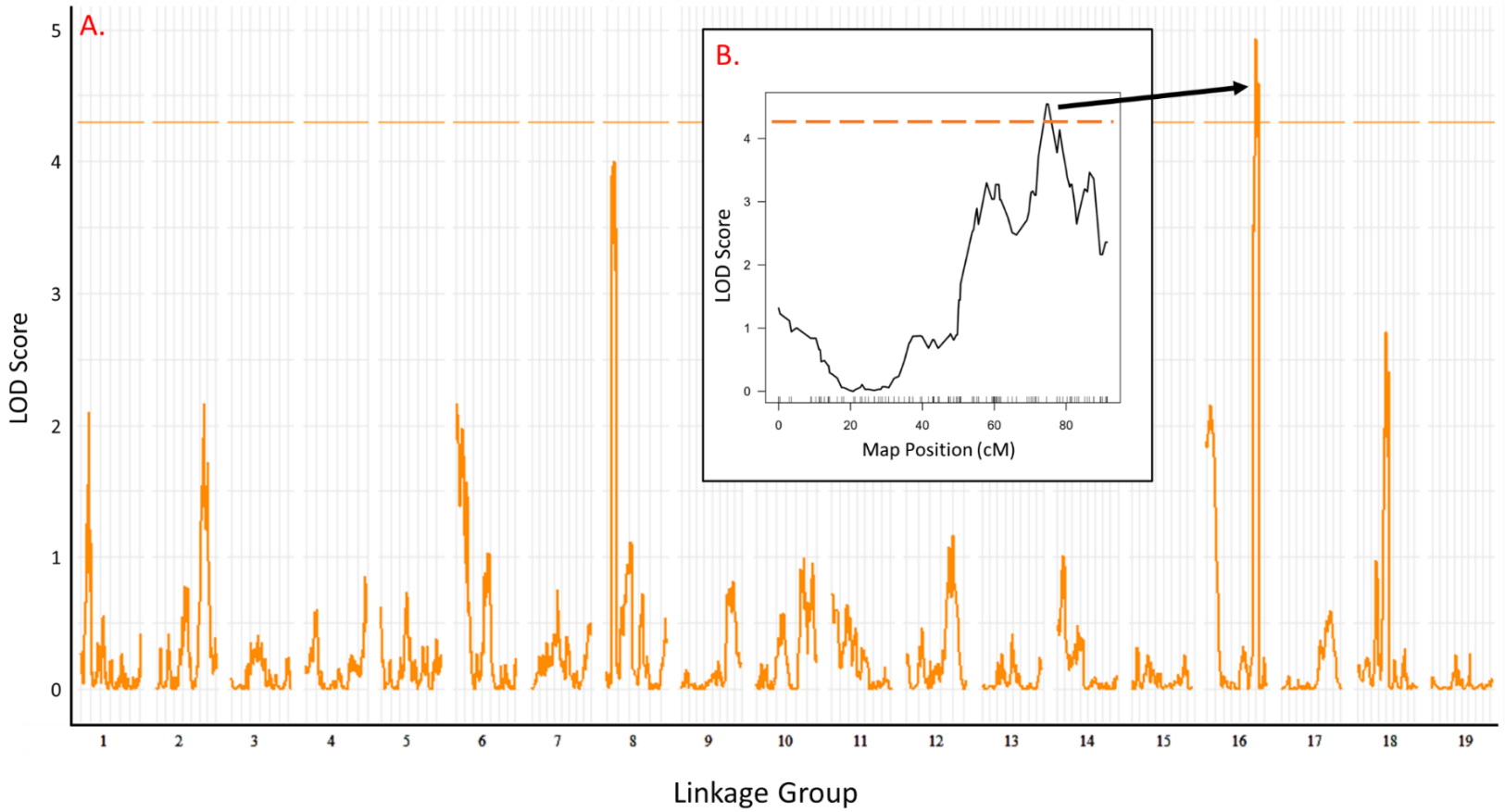

Fig. 2.4. A) The constructed QTL map from canker count is shown in orange. The X-axis shows the location of genes on the 19 genetic map linkage groups, corresponding to the 19 chromosomes of *Populus.* The Y-axis shows the LOD score associated with stem canker count phenotyping results as determined by R/qtl. α = 0.05 significance threshold shown where LOD = 4.3 (orange dashed line). **B)** Inset shows the Linkage Group 16 QTL peak in higher detail, where the Y-axis is the LOD score and the X-axis is the centiMorgan position of markers on the linkage group.

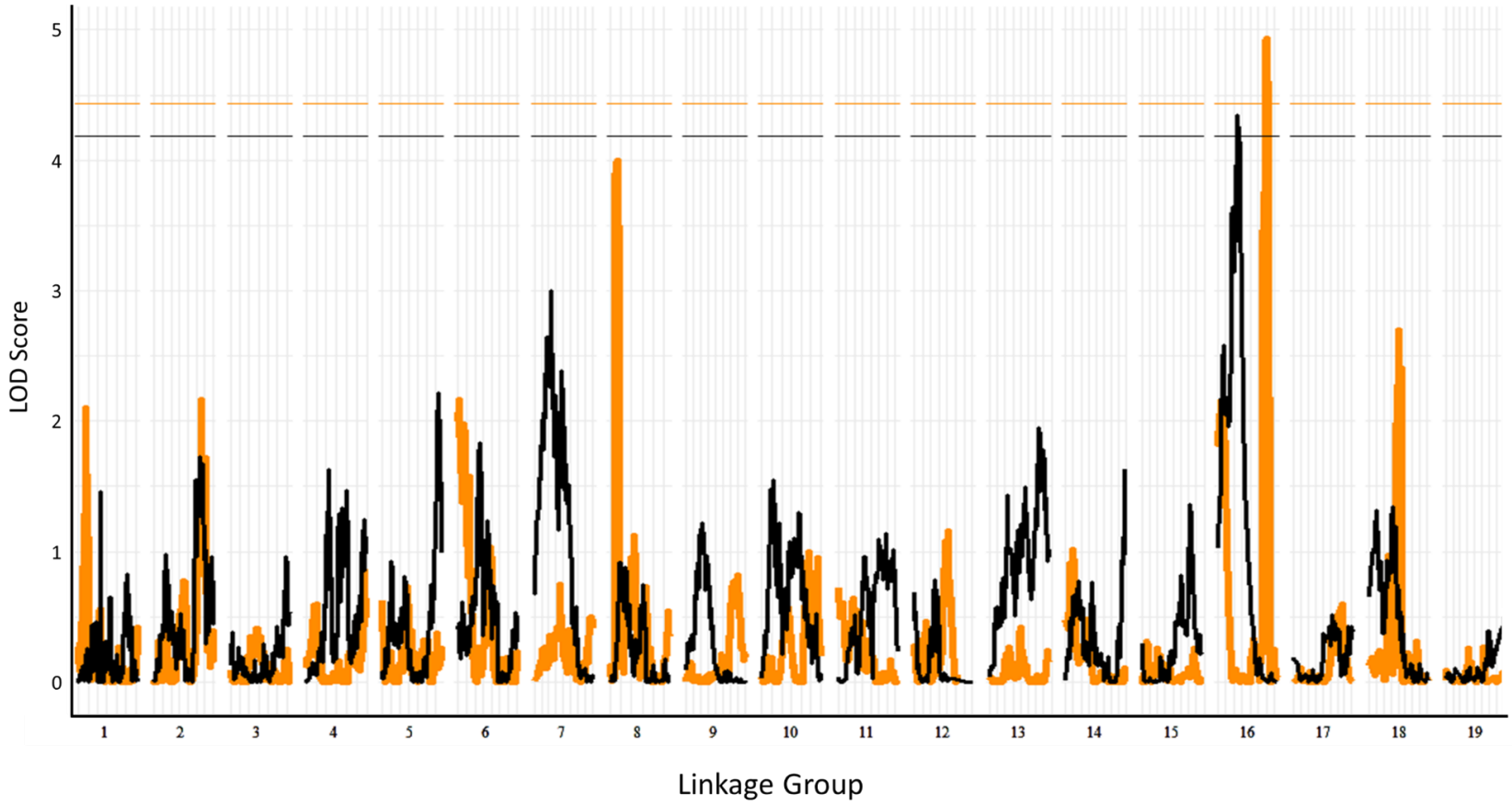

Fig. 2.5. Combined QTL plots from the greenhouse data reported in this greenhouse study (orange) and the field data reported in Simon *et al.* (unpublished; black).

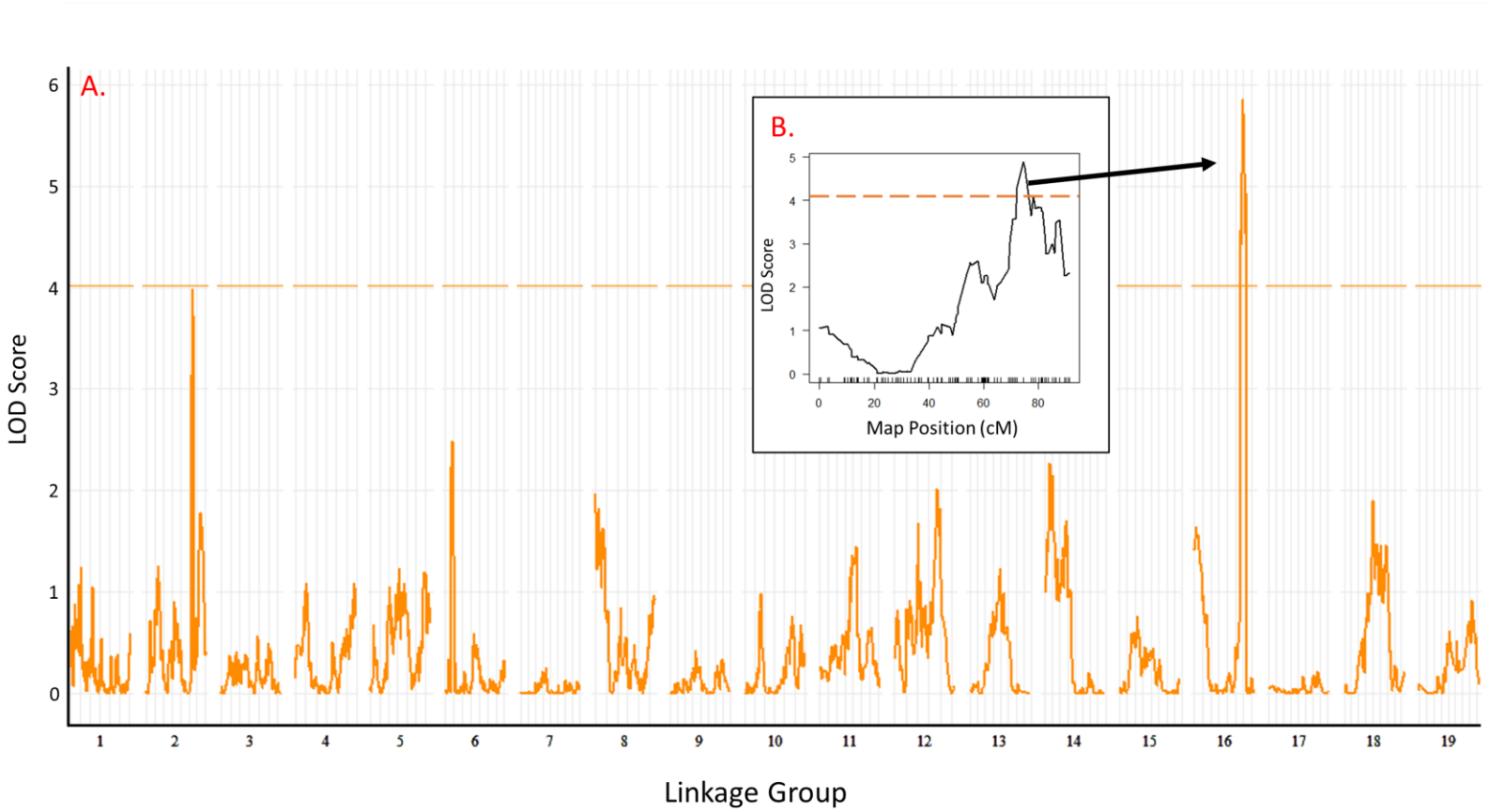

Fig. 2.6. A) The constructed QTL map from disease severity is shown in orange. The X-axis shows the location of genes on the 19 genetic map linkage groups, corresponding to the 19 chromosomes of *Populus.* The Y-axis shows the LOD score associated with stem canker disease severity phenotyping results as determined by R/qtl. α = 0.05 significance threshold shown where LOD = 4.02 (orange dashed line). **B)** Inset showing the Linkage Group 16 QTL peak in higher detail, where the Y-axis is the LOD score and the X-axis is the centiMorgan position of markers on the linkage group.

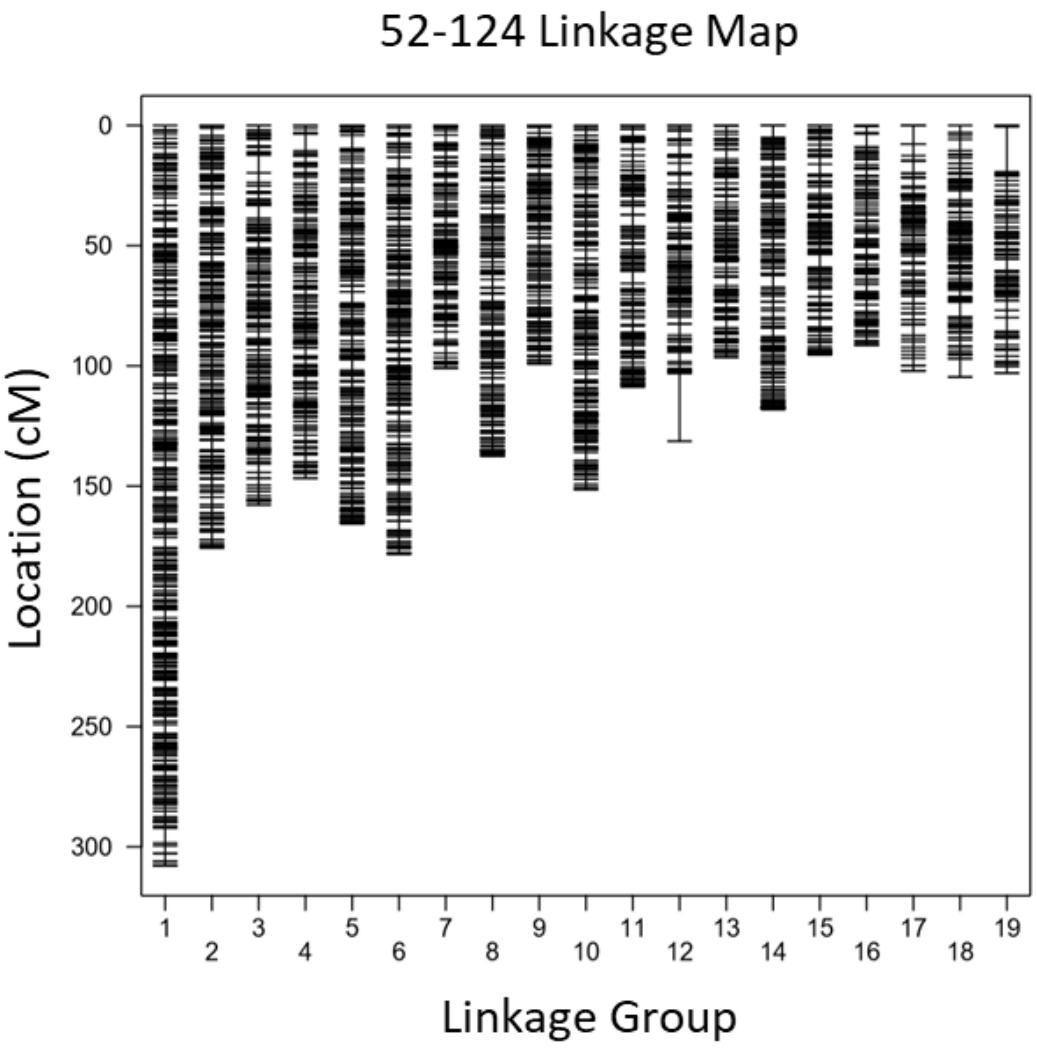

Fig. 2.7. Genetic linkage map coverage. The X-axis is the 19 linkage groups of the genetic map and the Y-axis is the centimorgan position on each linkage group. The presence of a marker is indicated by a solid, horizontal line and gaps between markers are visible as white spaces.

Table 2.1. Canker count phenotype data summary. Data is grouped by Block for the full dataset (284 genotypes) and the subset of data used for QTL modelling (230 genotypes), where *n* is the sample size of each block.

	Full Dataset					Data Subset				
	n	**Mean**	**Std. Dev.**	**Min.**	**Max.**	***n***	**Mean**	**Std. Dev.**	**Min.**	**Max.**
Block 1	277	4.43	8.26	0	46	227	4.67	8.62	0	46
Block 2	282	2.76	6.52	0	48	229	3.07	6.97	0	48
Block 3	272	6.83	10.90	0	61	220	6.77	11.00	0	61
Block 4	271	3.27	6.19	0	39	220	3.31	6.17	0	39
Block 5	265	9.10	12.80	0	78	215	9.34	13.00	0	78

Table 2.2. Disease severity phenotype data summary. Data is grouped by Block for the full dataset (284 genotypes) and the subset of data used for QTL modelling (230 genotypes), where *n* is the sample size of each block.

	Full Dataset					Data Subset				
	n	**Mean**	**Std. Dev.**	**Min.**	**Max.**	***n***	**Mean**	**Std. Dev.**	**Min.**	**Max.**
Block 1	277	2.09	1.39	1	5	227	2.08	1.38	1	5
Block 2	282	2.06	1.47	1	5	229	2.13	1.50	1	5
Block 3	272	2.36	1.57	1	5	220	2.30	1.55	1	5
Block 4	271	2.40	1.54	1	5	220	2.46	1.55	1	5
Block 5	265	2.38	1.38	1	5	215	2.42	1.37	1	5

Table 2.3. Segregation test for Septoria stem canker count phenotypes on TD × D F_2 pseudo-backcross hybrids in a greenhouse inoculation experiment. Tests were based upon a hypothesis of a putative, recessive resistance gene control under Mendelian genetics by Chi-Square Goodness-of-Fit with a Yates correction applied. Individuals were considered resistant if canker count = 0 and susceptible otherwise.

	Phenotype (Resistant : Susceptible, R:S)		
	Expected[a]	Observed	X^2 probability[b]
Before Map Filtering	683.5 : 683.5	604 : 763	$p < 0.0001$
After Map Filtering	555.5 : 555.5	474 : 637	$p < 0.00001$

[a] 1:1 R:S ratio was expected for this TD × D pseudo-backcross family.

[b] With Yates Correction and DF = 1

Table 2.4. Segregation test for Septoria stem canker disease severity phenotypes on TD × D F_2 pseudo-backcross hybrids in a greenhouse inoculation experiment. Tests were based upon a hypothesis of a putative, recessive resistance gene control under Mendelian genetics by Chi-Square Goodness-of-Fit with a Yates correction applied. Individuals were considered resistant if disease severity = 1 and susceptible otherwise.

	Phenotype (Resistant : Susceptible, R:S)		
	Expected[a]	Observed	X^2 probability[b]
Before Map Filtering	683.5 : 683.5	699 : 668	$p = 0.417$
After Map Filtering	555.5 : 555.5	555 : 556	$p = 0.952$

[a] 1:1 R:S ratio was expected for this TD × D pseudo-backcross family.

[b] With Yates Correction and DF = 1

Table 2.5. Variance estimates, estimated percent variance, and estimated p-values from random effect contributions for the canker count negative binomial GLMM not including any marker effects.

Source of Variation	Variance Estimate	Percent Explained Variance	Estimated p-value ($\alpha = 0.05$)
Genotype	0.4682	42.25%	$p < 0.0001$
Rack(Block)	0.2473	22.45%	$p < 0.0001$
Block	0.3926	35.43%	$p < 0.0001$
Total Explained	1.1081	100%	

Table 2.6. Variance estimates, estimated percent variance, and estimated p-values from random effect contributions for the canker count negative binomial GLMM including a fixed effect for allele type at the significant QTL on Linkage Group 16.

Source of Variation	Variance Estimate	Percent Explained Variance	Estimated p-value ($\alpha = 0.05$)
Genotype	0.3875	36.86%	$p < 0.0001$
Rack(Block)	0.2616	24.88%	$p < 0.0001$
Block	0.4022	38.26%	$p < 0.0001$
Total Explained	1.0513	100%	

Table 2.7. Variance estimates, estimated percent variance, and estimated p-values from random effect contributions for the disease severity LMM not including any marker effects.

Source of Variation	Variance Estimate	Percent Explained Variance	Estimated P-Value ($\alpha = 0.05$)
Genotype	0.2536	13.90%	$p < 0.0001$
Rack(Block)	0.0955	5.23%	$p < 0.0001$
Block	0.0590	3.23%	$p < 0.0001$
Residual	1.4166	77.63%	
Total Explained	1.8247	100%	

Table 2.8. Variance estimates, estimated percent variance, and estimated p-values from random effect contributions for the disease severity LMM including a fixed effect for allele type at the significant QTL on Linkage Group 16.

Source of Variation	Variance Estimate	Percent Explained Variance	Estimated P-Value ($\alpha = 0.05$)
Genotype	0.2034	11.44%	$p < 0.0001$
Rack(Block)	0.1047	5.89%	$p < 0.0001$
Block	0.0576	3.24%	$p < 0.0001$
Residual	1.4119	79.43%	
Total Explained	1.7776	100%	

Table 2.9. Selected candidate genes. Genes presented are a subset of the genes within the 1000 kb window of orthologues with (Ka/Ks) > 1 and predicted functions related to plant immunity, and non-orthologous genes with predicted functions related to plant immunity.

	Phytozome Gene Models		BLASTP		*Arabidopsis*-based gene
	P. trichocarpa	*P. deltoides*	E-score	(Ka/Ks)	annotation
Orthologous Genes	Potri.016G121600	Podel.16G127600	2E-32	4.70	*Salix* homologue (SapurV1A.0905s00): transmembrane protein, putative
	Potri.016G122700	Podel.16G128900	3.00E-81	3.14	Concanavalin A-like L-type lectin protein kinase family protein
	Potri.016G125800	Podel.16G131100	9E-39	2.31	BRCA2-like B
	Potri.016G126800	Podel.16G132200	4.00E-71	1.46	Tautomerase/MIF superfamily protein
	Potri.016G129400	Podel.16G135200	4.00E-81	1.12	hydroxyproline-rich glycoprotein family protein
P. deltoides **non-orthologous genes**	--	Podel.16G132500	--	--	receptor like protein 6
	--	Podel.16G125800	--	--	receptor like protein 7
	--	Podel.16G126100	--	--	receptor like protein 19
	--	Podel.16G125900	--	--	receptor like protein 33
	--	Podel.16G131900	--	--	Tautomerase/MIF superfamily protein
P. trichocarpa **non-orthologous genes**	Potri.016G127000	--	--	--	receptor like protein 6
	Potri.016G127100	--	--	--	receptor like protein 33

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Chapter 3: Protocol for Development and Screening of *S. musiva* Transformants

3.1. Introduction

The virulence of plant pathogens may be affected by a variety of factors, including gene content. The multifaceted basis behind a plant disease epidemic was recognized as early as the 1900s, when Duggar (1909) wrote about the connection between environmental conditions and pathogenicity, including factors such as ideal temperatures for fungal sporulation and the effect of light availability on plant pathogenic bacteria. The disease triangle built upon these observations as a paradigm suggesting that a susceptible host, an infectious pathogen, and a suitable environment are all contributors to a plant disease epidemic (Drenth 2004). Suggested adjustments to the disease triangle have intended to account for perceptions of unequal contributions from the host, pathogen, and environment, or additional contributing factors such as time (Francl 2001). Still, the disease triangle paradigm remains influential to the present day and likely drives investigations of the host, pathogen, and environment in plant pathology (David et al. 2019; Ireland and Kriticos 2019; Liu and He 2019; Simler et al. 2019). Enabling the genetic transformation of pathogens would allow the characterization of the role of the pathogen in disease.

Allelic variation in species of *Populus* has been exploited through natural breeding efforts and plant transformation (Cram 1960; Einspahr and Benson 1964; Tuskan et al. 2006; Atiq et al. 2019; Miller et al. 2019; SriBala et al. 2019). While the impact of environmental factors may be minimized by conducting studies in controlled environments, researchers are just beginning to understand the role of pathogen-derived molecules such as secreted proteins typically called effectors (de Sá 1992; Martin et al. 2003; Wang and Constabel 2004; Jones and Dangl 2006; de Jonge et al. 2011; Henry et al. 2012; Plett 2014; Dunnell 2016).

Jones and Dangl (2006) described the role of fungal effectors in their Zig-Zag model, which outlines the evolutionary arms race between plant pathogens and their hosts. Briefly, the first layer of plant defense involves the recognition of broadly conserved molecular motifs belonging to plant pathogenic organisms, called pathogen-associated molecular patterns (PAMPs; Jones and Dangl 2006; Henry et al. 2012; Macho and Zipfel 2014). These PAMPs are recognized by an extracellular domain of a membrane-bound protein called a pattern recognition receptor (PRR). PAMP recognition leads to cellular responses and downstream signaling which initiates PAMP-triggered immunity (PTI; Macho and Zipfel 2014; Kushalappa et al. 2016). A PTI-response often causes a change in gene transcription and activation of MAP kinase signaling (Chinchilla et al. 2006; Chisholm et al. 2006; Jones and Dangl 2006).

In order to suppress PTI, fungal pathogens have evolved effectors (Jones and Dangl 2006), which are typically small (M_w < 20 kilodaltons) secreted proteins, that interact with the host plant at hyphal tips or via haustoria (de Jonge et al. 2011). Different mechanisms of effector activity have been identified, including effectors binding to PAMPs to impede PRR activity and the disruption of cell signaling events (Shan et al. 2008; de Jonge et al. 2010; de Jonge et al. 2011; Sánchez-Vallet et al. 2012; Zhang et al. 2012). In response to fungal effectors, hosts are thought to have evolved *R* genes. The gene products encode nucleotide-binding site (NBS) domains and leucine-rich repeat (LRR) domains that recognize effectors directly or indirectly, triggering localized programmed cell death (Jones and Dangl 2006; Kushalappa et al 2016). This immune response to effectors is called effector-triggered immunity (ETI; Jones and Dangl 2006; Kushalappa et al. 2016; Künstler et al. 2016).

Sphaerulina musiva (Peck) Quaedvl., Verkley & Crous (syn. = *Septoria musiva*) is an ascomycete native to the north-eastern regions of North America, where it causes leaf spot symptoms on its sympatric host, *Populus deltoides* (Bier 1939; Ostry 1987; Callan et al. 2007). On naïve species, including *Populus trichocarpa* and *P. trichocarpa* × *P. deltoides* (T × D) hybrids, symptoms may include both leaf spot and necrotic lesions on stems and branches which may compromise structural integrity and lead to tree mortality (Zalasky 1978; Ostry and McNabb 1985; Feau et al. 2010). Several studies have been conducted in order to identify the genes that confer resistance to *S. musiva*

(Muchero et al. 2015; Simon et al., unpublished; and Chapter 2). In order to fully understand and characterize the mechanisms of resistance, an understanding of the pathogen in essential.

Sphaerulina musiva effectors have recently been predicted by aligning RNAseq reads to the *S. musiva* genome and filtering fungal reads for low molecular weight (M_w < 20 kDa) proteins with predicted secretion signal cleavage sites and differential expression after inoculation of *P. trichocarpa* genotype GW-10998 with *S. musiva* isolate MN-14 (Dunnell 2016). In order to demonstrate the role of these effectors, the ability to manipulate the genome of the fungus is essential. Fungal gene disruption studies based upon *Agrobacterium tumefaciens*-mediated transformation (ATMT) have largely been successful in demonstrating the role of fungal effectors in *Aspergillus fumigatus*, *Verticillium dahliae*, and *Cladosporium fulvum,* among others (Dobinson et al. 2004; Sugui et al. 2005; Wang et al. 2016). This approach has also been used with *S. musiva*, where a polyketide synthase-like gene (*PKS-L1*) was disrupted and fluorescent protein sequences have been inserted (Foster et al. 2014; Abraham 2019). In this example, gene disruption was facilitated by homologous recombination within a cell.

In general, ATMT-mediated gene disruption in *S. musiva* uses the following approach. *Sphaerulina musiva* disruption plasmids are designed to have an antibiotic resistance gene flanked by homologous sequences corresponding to the 5’ and 3’ end of the target gene (Fig. 3.1; Khang et al. 2006; Foster et al. 2014; Abraham et al. 2019). This

disruption cassette is centered between the left border (LB) and right border (RB) of the T-DNA in an *A. tumefaciens*-compatible binary vector. Following co-cultivation of *A. tumefaciens* cells and germinating fungal spores, the T-DNA is inserted into the fungal cells (Khang et al. 2006; Foster et al. 2014; Abraham et al. 2019). Subsequently, homologous recombination disrupts the target gene by integrating the resistance cassette into the fungal genome (Fig. 3.1; Khang et al. 2006; Foster et al. 2014; Schuster et al. 2015; Abraham et al. 2019). The final product is a disrupted gene wherein the genetic space between the homologous flanking (5' and 3') sequences is replaced with the antibiotic resistance gene.

Despite the availability of published literature on successful transformation of filamentous fungi, transformation is technically difficult. The specific objectives were to transform *S. musiva* with two different binary vectors: 1) one designed to randomly disrupt genes within the *S. musiva* genome (pPL1); and 2) a second designed to disrupt a specific gene (KO-nrps1).

3.2. Materials and methods

3.2.1. Binary plasmids

Two plasmids were used in this study, provided by Dr. Philippe Tanguay from the Natural Resources Canada (NRCan) Laurentian Forestry Centre (LFC; Foster et al., 2014). The first plasmid (pPL1) was a binary vector with a hygromycin resistance cassette

designed for random insertion into the *S. musiva* genome. The second binary vector (KO-nrps1) was designed for disruption of *S. musiva* gene *nrps1* (Sm134219) by homology-mediated Hygromycin resistance cassette integration. Plasmids were received as circular DNA dehydrated onto filter paper inside a marked circle. Plasmid DNA was reconstituted by cutting the circle out with sterile scissors and transferring the piece of filter paper to a sterile 1.5-mL microcentrifuge tube. To this tube, 100 µL of TE buffer (1 M Tris-Cl, pH 8.0; 0.5M EDTA, pH 8.0) was added. The tube was vortexed briefly before incubation at room temperature for 5 min. The tube was vortexed a second time, centrifuged for 5 sec at 5000 rpm and stored at -20 °C. In addition to the Hygromycin resistance cassette, the pPL1 plasmid contained an antibiotic resistance gene for Kanamycin, and the KO-nrps1 plasmid contained an antibiotic resistance gene for Spectinomycin. Final concentrations of Kanamycin and Spectinomycin on selective media were always prepared at 50 mg/L and 100 mg/L, respectively.

3.2.2. Transformation of *E. coli*

For each construct (pPL1 and KO-nrps1), a single tube of chemically competent *E. coli* cells (10-β High Efficiency Competent®; New England Biolabs, Ipswich, MA, U.S.) were thawed on ice. To each tube of competent cells, 10 µL of reconstituted plasmid DNA was added. The contents of the tube were gently mixed and incubated for 30 min on ice. The mixture was heat shocked at 42 °C for 30 sec without mixing and placed on ice for 5

minutes. An additional 950 µL of Stable Outgrowth Medium (New England Biolabs, Ipswich, MA, U.S.) was added to the mixture. Each tube was transferred to a 37°C shaking incubator for 60 minutes at 250 rotations per minute (rpm). After incubation, the cell suspension was gently mixed and two LB agar (Becton-Dickinson, BD/Difco, Sparks, MD, U.S.) cultures amended with Kanamycin or Spectinomycin were prepared for each construct: 1) a 50 µL of cell suspension; and 1) a 100 µL cell suspension. Plates were incubated overnight at 37°C. Single colonies (n = 3) of *E. coli* were selected for each construct using a sterile pipette tip and grown in overnight cultures consisting of 5 mL of LB liquid media (Becton-Dickinson, BD/Difco, Sparks, MD, U.S.) amended with Kanamycin or Spectinomycin. Plasmid DNA was extracted from all overnight cultures using the QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. A 1.0-ml subsample of each culture was stored in 25% glycerol at -80°C.

3.2.3. Transformation of *A. tumefaciens*

Agrobacterium tumefaciens cells (strain EHA105; OD_{550} ~ 0.5-0.8) were removed from a -80°C freezer and allowed to thaw on ice. Purified plasmid DNA (100-1000 ng), extracted from *E. coli,* was added to 50 µL of EHA105 cells. One of the three purified plasmid DNA samples was used for each binary vector. The solution was gently mixed and incubated for 25 min on ice. The cell suspension was submerged in liquid nitrogen for 2 minutes,

followed by a 5-minute heat treatment in a 37°C water bath. The mixture was cooled on ice for 5 min and 0.7 mL of YEP media (MP Biomedicals, Irvine, CA, U.S.) was added. The transformation mixture was incubated for 1-2 hours at 28°C. Incubation was followed by serial dilution (1, 1:10, and 1:100) and plating on LB selection plates amended with Kanamycin or Spectinomycin. After 3-4 days of growth at room temperature, colonies were visible. Overnight cultures were made in LB media amended with Kanamycin or Spectinomycin, and a 1.0-mL subsample of each culture was stored in 25% glycerol at -80°C.

3.2.4. Transformation of *S. musiva*

Sphaerulina musiva isolate MN-14 was transformed using the following procedure adapted from Foster *et al.* (2014) and Khang *et al.* (2006). All media were prepared as outlined in Khang *et al.* (2006). Stock solutions were prepared in total volumes of 100 mL of deionized H_2O: K-buffer (pH 7.0) [20 g K_2HPO_4; 14.5 g KH_2PO_4], M-N Buffer [3 g $MgSO_4 \cdot 7H_2O$; 1.5 g NaCl], a 1% $CaCl_2 \cdot H_2O$ Solution [1 g $CaCl_2 \cdot H_2O$], Spore Element Buffer [0.01 g $ZnSO_4 \cdot 7H_2O$; 0.01 g $CuSO_4 \cdot 5H_2O$; 0.01 g H_3BO_3; 0.01 g $MnSO_4 \cdot H_2O$; 0.01 g $Na_2MoO_4 \cdot 2H_2O$], a 20% NH_4NO_3 solution [20 g NH_4NO_3], a 20% Glucose solution [20 g Glucose], a 0.01% $FeSO_4$ solution [0.01 g $FeSO_4$], a 50% Glycerol solution [50 mL Glycerol], and a 1M MES solution (pH 5.3) [21.32 g MES]. Each solution was autoclaved separately before mixing, excluding the glycerol, $FeSO_4$, Kanamycin or Spectinomycin,

and MES solutions, which were sterilized by filtration through a 0.22-µm filter. To prepare the following media, all autoclave-sterilized solutions were mixed, autoclaved, and cooled to 70°C prior to adding filter-sterilized reagents.

Minimal media (MM) consisted of 1 mL of K-Buffer (pH 7.0), 2 mL of M-N Buffer, 0.1 mL of 1% $CaCl_2 \cdot H_2O$ solution, 1 mL of Spore Element Buffer, 0.25 mL of 20% NH_4NO_3 solution, 1 mL of 20% Glucose solution, 1 mL of 0.01% $FeSO_4$ solution, 0.15 mL of Kanamycin (50 mg/mL) or Spectinomycin (100 mg/mL), and 93.5 mL of sterile H_2O. Induction media (IM) consisted of 1 mL of K-Buffer (pH 7.0), 2 mL of M-N Buffer, 0.1 mL of 1% $CaCl_2 \cdot H_2O$ solution, 1 mL of Spore Element Buffer, 0.25 mL of 20% NH_4NO_3 solution, 1 mL of 20% Glucose solution, 1 mL of 0.01% $FeSO_4$ solution, 1 mL of 50% Glycerol solution, 4 mL of 1M MES (pH 5.3), 0.15 mL of Kanamycin (50 mg/mL) or Spectinomycin (100 mg/mL), 0.2 mL Acetosyringone (200 mM in 95% ethanol), and 88.5 mL of sterile H_2O. Co-cultivation media (CM) was identical to IM but had 1.5 g of agar added prior to autoclaving.

Overnight 1-mL cultures of transformed *A. tumefaciens* were made in MM amended with Kanamycin or Spectinomycin and grown for 48 hours at 28°C until the optical density at 600nm (OD_{600}) was 0.6. The samples were then diluted to $OD_{600} = 0.15$ with approximately 6-8 mL of IM. Each sample was split into two equal volumes (3-4 mL) to facilitate growth. Samples were incubated in a shaking incubator at 250 rpm and 28°C until $OD_{600} = 0.6$. Conidia were harvested from 8-day-old cultures of MN-14 grown on

potato dextrose agar (Becton-Dickinson, BD/Difco, Sparks, MD, U.S.) by adding 1 mL of nuclease-free (NF) water and gently rubbing the surface of the plate with a sterile inoculation loop. The concentration of conidia was determined using a hemocytometer. Cells of *A. tumefaciens* and conidia of *S. musiva* were then combined at 1:1 concentration and ca. 2 x 10^7 conidia were spread onto sterile 47-mm PVDF membrane discs with a 0.45-µm pore size (Pall Corp, Port Washington, NY, U.S.). The membranes were placed on the surface of cocultivation media (CM) plates.

The cultures were incubated in the dark for 60 h at room temperature. The germination rate of spores on the membranes was not estimated. Membranes were transferred from CM plates to selective oatmeal agar (OMA; Becton-Dickinson, BD/Difco, Sparks, MD, U.S.) plates amended with hygromycin (50 µg/mL), cefotaxime (200 µg/mL), and moxalactam (100 µg/mL). Membranes were removed from the CM plates, flipped culture-side-down onto the selective media surface, and incubated for 5 min. To remove bacteria, the membranes were gently slid across the OMA media surface, placed culture-side-up, and incubated at room temperature in the dark for 21 d. Membranes with visible colonies were transferred to new OMA plates and grown for an additional 7 days. 1 mL of nuclease-free water was added to each plate, and the melanized cultures were gently scraped with a sterile inoculation loop. This suspension was then plated on OMA selection plates. Both the original membrane plate and the new non-membrane plate were grown in the dark until sporulation was visible.

3.2.5. Single-spore isolations

All microscopy images were used for qualitative visualization, total magnification was not recorded, and images were taken using a Nikon DS-Fi2 camera running the NIS-Elements F software (ver. 4.00.00, Build 764; Nikon, Minato, Tokyo, Japan). After sporulation, single spore isolations were prepared and plated (Zhang et al. 2013). Two water agar plates were prepared for each transformant. A 10 x 10 mm square grid was drawn on the back of each plate. Two pycnidia were excised from a sporulating colony on the non-membrane OMA plates and placed in 2 microcentrifuge tubes. One tube contained 200 µL of nuclease-free water and the other tube contained 400 µL of nuclease-free water. A single drop (~ 5 µL) from one of the spore suspensions was plated inside each square. One plate was assigned the 200-µL suspension, and the other plate was assigned to the 400-µL suspension. Plates were allowed to dry, wrapped in parafilm, and incubated in the dark at 25°C for 6 hours. Plates were then examined with a dissecting microscope (Nikon SMZ18 Stereoscope) for germinating spores. Individual spores were extracted from the water agar plate using a sterile, 26-gauge needle. Between 4-5 individual spores were plated onto a large K-V8 agar plate [180 mL V8 juice (Campbell Soup Company, Camden, NJ, U.S.), 2 g calcium carbonate, 20 g bacteriological agar, and 820 mL deionized water] supplemented with Streptomycin sulphate (100 mg/L) and Chloramphenicol (246 mg/L). The plates were wrapped in parafilm, and

grown in the dark at 25°C. Once mycelial growth was visible, each colony was sub-cultured onto a new K-V8 plate without antibiotics. Plates were incubated for approximately 2 weeks at room under continuous, full-spectrum fluorescent bulbs (Sylvania, Wilmington, MA, U.S.; Osram Gmbh, Munich, Germany) until sporulation was observed. For each putative transformant, approximately three 2 x 5 mm plugs of sporulating single-spore cultures were transferred to cryogenic tubes, vortexed briefly, and stored at -80 °C in a 50% glycerol solution.

3.2.6. DNA extraction and PCR screening

DNA was extracted from single-spore cultures for PCR confirmation of successful transformation. Initially, three small plugs (ca. 2 x 5 mm) of sporulating mycelium from each transformant were placed in 100 ml of K-V8 liquid medium [820mL of NanoPure water, 180mL of V8 juice (Campbell Soup Company, Camden, NJ, U.S.), 2g of Calcium Carbonate, and no antibiotics] in 250-ml Erlenmeyer flasks. Cultures were shaken (150 rpm) at room temperature in the dark for 5-7 days. After incubation, liquid cultures were then poured into a funnel lined with Miracloth (MilliporeSigma, Burlington, MA, U.S.). Excess liquid media was removed by gently squeezing the Miracloth. Approximately 75 mg of mycelium was placed in a KingFisher Flex-compatible magnetic bead tube and sent to the Center for Genome Research and Biocomputing (CGRB) Core

Facilities at Oregon State University for automated DNA extraction using the Mag-Bind® Plant DNA DS 96 Kit protocol (Omega Bio-tek, Norcross, GA, U.S.).

Both the pPL1 and KO-nrps1 putative transformants were screened by PCR. Initially, the INTEG_FOR3 and pPGW_HygR_REV1 primer set were designed to amplify a small, 783-bp portion of the Hygromycin resistance cassette (Fig. 3.2; Table 3.1). Putative KO-nrps1 transformants were screened further with the pPGW_HygR_FOR1 and pPGW_HygR_REV1 primer set designed to amplify a 1392-bp amplicon accounting for approximately 96.5% of the entire 1443-bp hygromycin resistance cassette (Fig. 3.3; Table 3.1). Finally evidence for disruption of the *nrps1* (Sm134219) gene in MN-14 was evaluated by using a third pair of primers (NRPS1_WT_FOR2 and NRPS1_WT_REV3; Table 3.1) which amplified a 2136-bp portion of *nrps1* genomic DNA that was expected to be displaced by the hygromycin resistance cassette. Presence of a 2136 bp band on an agarose gel would indicate a failed site-specific transformation (Fig. 3.4). All PCR products were visualized on a 1% agarose gel stained with 1X GelRed dye (Biotium, Hayward, CA, U.S.). Imaging was performed on an Azure c200 Gel Imaging System (Azure Biosystems, Dublin, CA, U.S.) at 302 nm with a 10 s exposure time.

3.3. Results

Conidial germination rate was not evaluated in this study. The PVDF membranes were opaque and it was not possible to see the spores using a compound scope. However,

germinating conidia were evident in the co-cultivation media after 60 hours (Fig. 3.5A). For similar reasons, transformation efficiency was not estimated. However, compound microscopy provided evidence of mycelial growth after plating on selective oatmeal agar plates after two days (Fig. 3.5B). Images of single spore cultures plated on fresh K-V8 plates taken by dissection microscopy were used to qualitatively assess the success of single spore isolates in developing into mycelial colonies (Fig. 3.6A-D).

Transformation success was evaluated using PCR to screen extracted genomic DNA from both putatively transformed and wild type MN-14. All random insertion (pPL1) transformants (5/5) showed evidence of hygromycin resistance cassette integration (Fig. 3.7, Lanes 3-7). Similarly, 4/5 directed disruption (KO-nrps1) mutants, screened by PCR, showed evidence of integration of the same 783-bp portion of the hygromycin resistance cassette (Fig. 3.7, Lanes 10-15). Amplification of the 1392-bp PCR product corresponding to 96.5% of the hygromycin resistance cassette had similar PCR results for all but one of the *nrps1*-directed disruption mutants (Fig. 3.8, Lanes 4-11). The 783-bp PCR negative transformant was the same mutant that failed the 1392-bp PCR screen. Genomic DNA extracted from WT MN-14 did not have bands corresponding to the 783 bp or the 1392 bp PCR product (Fig. 3.7, Lane 9). However, the final destination of the hygromycin resistance cassette in the *nrps1*-directed mutants could not be determined using these first two PCR screening assays. The third PCR screen was designed to amplify the portion of the *S. musiva nrps1* gene that would be replaced by

the hygromycin resistance cassette, indicating a failed gene disruption. All putative transformants had a band approximately 2136 bp in size, indicating failure of *nrps1* gene disruption (Fig. 3.9, lanes 4-11). Furthermore, this PCR product corresponded with the size of the band produced by the wild-type, untransformed MN-14 genomic DNA (Fig. 3.9, lane 12).

3.4. Discussion

To date, at least two different studies have successfully transformed the fungal pathogen *S. musiva* using the ATMT approach (Foster et al. 2014; Abraham et al. 2019). This study was also successful. A total of 5/5 pPL1 transformants appear to have been generated and 7/8 of the KO-nrps1 colonies appear to have been transformed, but with off-target effects. A successful *nrps1*-disruption was not detected for any putative KO-nrps1 transformants in this study (Fig. 3.5). However, two lines of evidence suggest that insertion of the Hygromycin resistance cassette was in fact successful: 1) colonies were capable of growing on OMA containing hygromycin whereas WT *S. musiva* MN-14 conidia did not develop mycelium on the OMA; 2) for each colony, an amplicon corresponding to the hygromycin resistance cassette was amplified (Figs. 3.3-3.4) and this amplicon was absent from the WT MN-14. In the future a larger number of putative transformants could be screened in a similar manner.

Prior to the adoption of this protocol for screening large numbers of transformants, many improvements are recommended. First, while the PVDF (Pall Corp, Port Washington, NY, U.S.) filter membranes used in this experiment allowed transformed mycelium to grow and pycnidia to develop on selective media, they may lead to changes in *S. musiva* culture morphology (such as melanization), complicating germination rates and transformation efficiency calculations. Cellophane membranes, similar to those used by Foster *et al.* (2014), would likely avoid these complications. If the pore size of the membrane is of concern, the success of germination and mycelium development of wild-type, non-transformed *S. musiva* conidia plated on cellophane membrane laid over non-selective oatmeal agar is suggested. Additionally, it may be useful to determine the ideal concentration of Hygromycin on selective OMA plates by constructing an antibiotic resistance curve, as different concentrations of Hygromycin have been reported for selection in the literature (Foster et al. 2014; Abraham 2019). One putative transformant (KO-nrps1 transformant #6) appeared to have survived the transformation protocol despite consistently providing no evidence that the hygromycin resistance cassette was integrated into its genome (Figs. 3.7-3.9). However, a negative control (a culture of the wild-type MN-14 isolate of *S. musiva* which never came in contact with *A. tumefaciens*) did not grow on the selective media at the concentrations of Hygromycin used in this protocol. Based on this trial, escape events may be as high as 12.5%, reinforcing the need for careful screening of transformants.

A pair of primers capable of amplifying as much of the Hygromycin resistance cassette as possible in pPL1 transformants should be developed in order to improve PCR screening efficiency. Although site-directed gene disruption events are expected to occur at specific loci in the *S. musiva* genome based upon homology with flanking regions in the T-DNA, pPL1 transformant screening relies solely on the amplification of the hygromycin resistance cassette, making it extremely difficult to localize the site of gene disruption.

The development of an accurate and consistent colony PCR protocol may help to both expedite transformant screening and reduce associated DNA extraction costs. For colonies which appear to be successful transformants based upon PCR screening results, additional steps such as amplicon sequencing may assist in identifying transformants with off-target transformation or unexpected frame-shift mutations. Based upon the results observed, eight putative KO-nrps1 transformants were not sufficient to generate one with the correct disruption of the *nrps1* gene. Personal communication with Dr. Philippe Tanguay at the Laurentian Forestry Centre with the Canadian Forest Service revealed one hundred or more putative transformants may need to be screened to find a single disruption mutant.

The disruption of fungal genes has potential to help characterize the interactions between pathogen effectors and host responses in the *Sphaerulina-Populus* pathosystem. Two different types of transformation events were attempted: 1) random

gene disruption (pPL1), and 2) specific gene disruption (KO-nrps1). Although the disruption of random genes within the *S. musiva* genome appears to have been successful, the disruption of the *nrps1* gene was not.

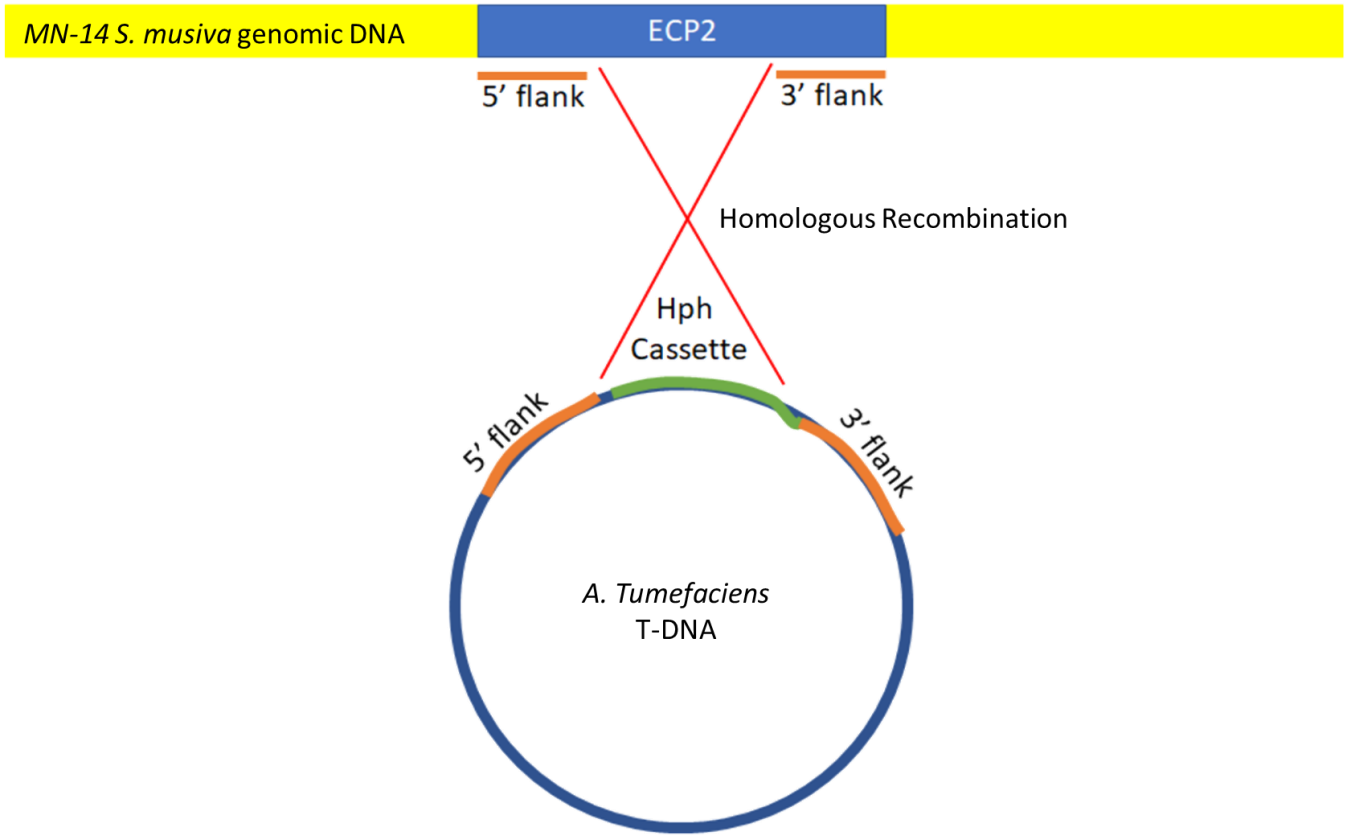

Fig. 3.1. A simple representation of how gene-directed disruption of a putative effector gene product, ECP2, may work under the *Agrobacterium tumefaciens*-mediated transformation (ATMT) protocol.

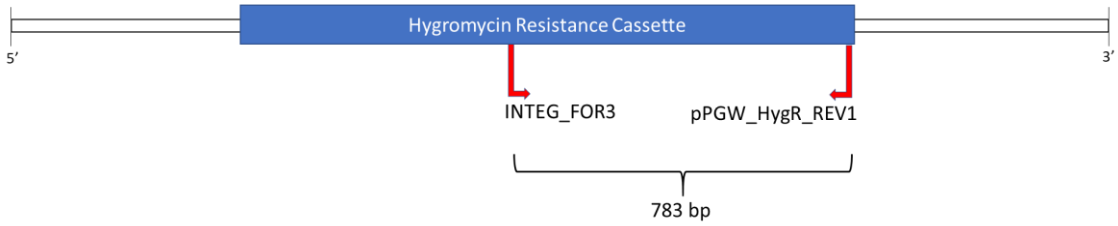

Fig. 3.2. PCR primers used to screen putative random insertion transformants for a 783-bp portion of the hygromycin resistance cassette. Locations shown are approximate.

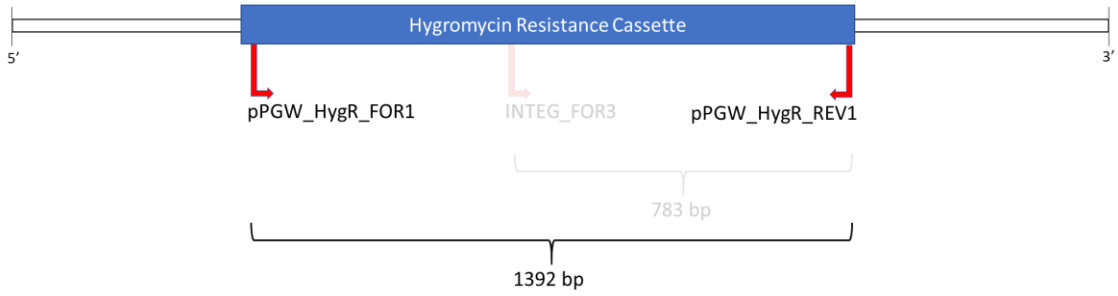

Fig. 3.3. PCR primers used to screen putative directed disruption transformants for a 1392-bp portion (96.5%) of the entire hygromycin resistance cassette. Locations shown are approximate and the approximate locations of the related 783-bp primer (INTEG_FOR3) and its associated amplicon size can be seen in faded text and color.

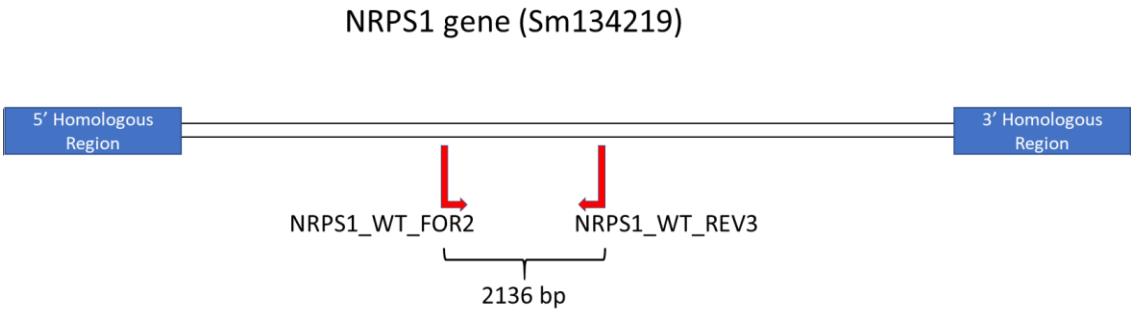

Fig. 3.4. PCR primers used to screen putative directed disruption transformants for a 2136-bp portion of the *nrps1* (Sm134219) gene. This genomic DNA is expected to be displaced (and not amplified) by homologous recombination in successful transformants based upon homology between 5' and 3' flanks in the genomic DNA of MN-14 and the T-DNA of transformed *Agrobacterium tumefaciens*.

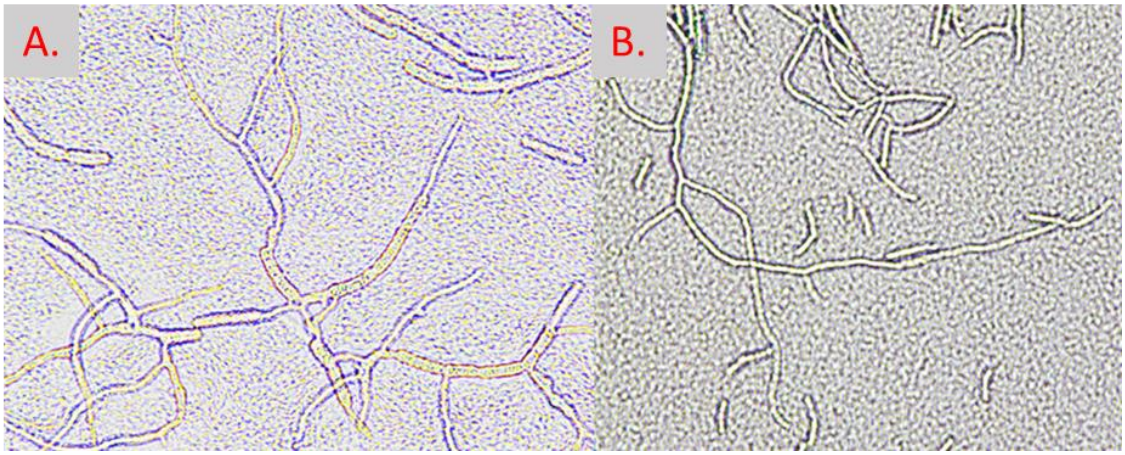

Fig. 3.5. Compound microscopy used for qualitative determination of germination and mycelium development. Images of **A)** germinating spores of putative *nrps1*-directed disruption transformant after 60 hours on co-cultivation media plates, and **B)** developing mycelium taken from a sample of putative KO-nrps1 transformant after two days on selective oatmeal agar media plates. Total magnification was not recorded.

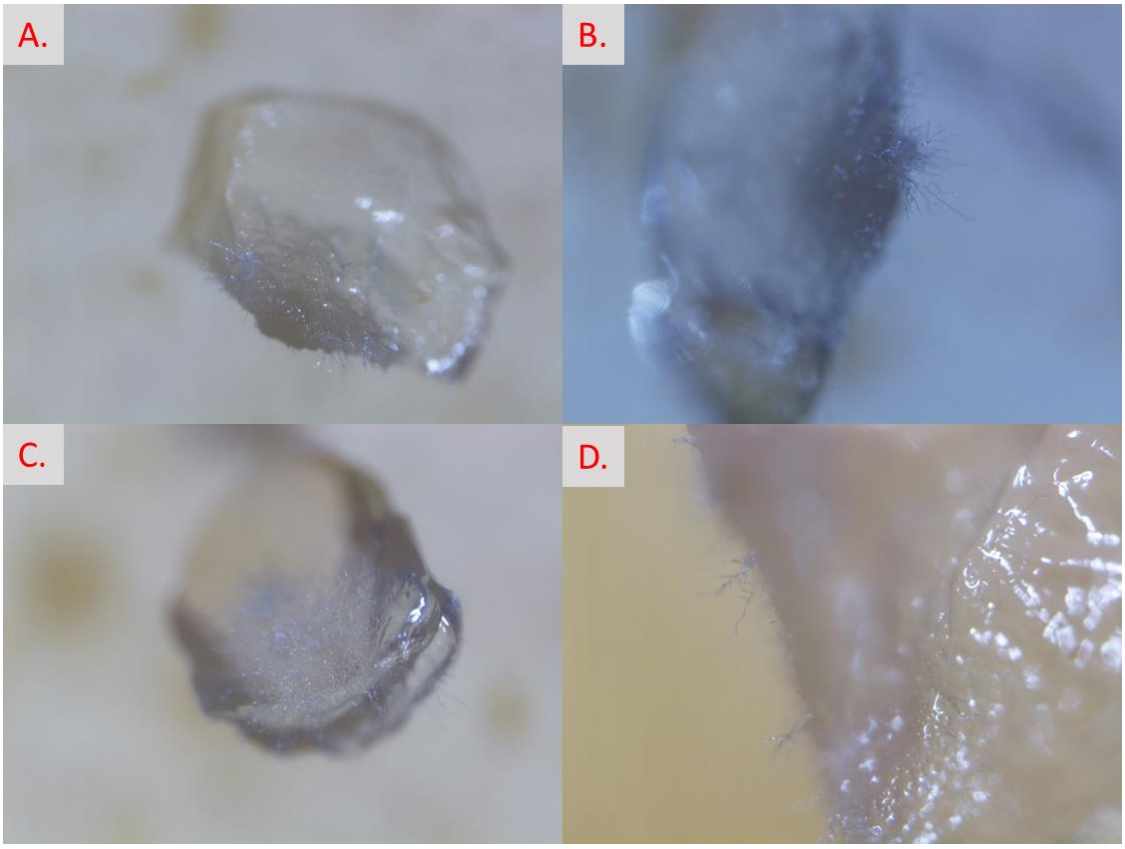

Fig. 3.6. Dissection microscopy of single-spore isolates. Images were used to qualitatively assess the success of four different putative KO-nrps1 transformants to colonize K-V8 media plates and form mycelium from single-spore isolates. Total magnification was not recorded.

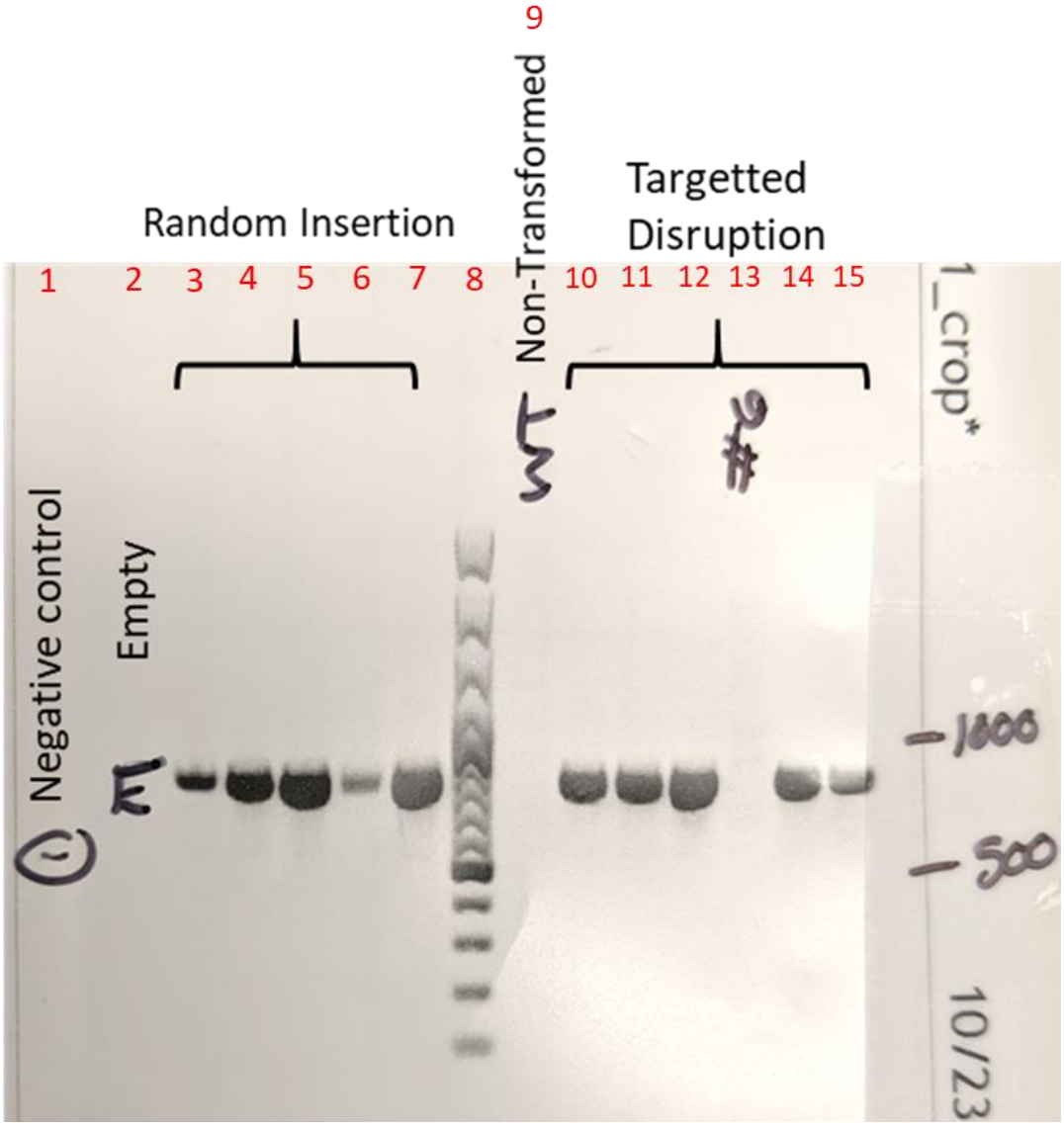

Fig. 3.7. PCR screen results for a 783-bp portion of the Hygromycin resistance cassette. Products were run on a 1.0% agarose gel to evaluate evidence of partial (783/1443 bp) Hygromycin resistance cassette integration into *S. musiva* isolate MN-14 genomic DNA by random insertion (lanes 3-7) or directed disruption (lanes 10-15). Lane Identity: 1 = Negative Control, 2 = Empty, 3-7 = Putative Random Insertion Transformants, 8 = 100bp plus Gene Ruler (Ready-to-Use), 9 = WT MN-14 (Non-Transformed), 10-15 = Putative Directed *nrps1* Disruption Transformants.

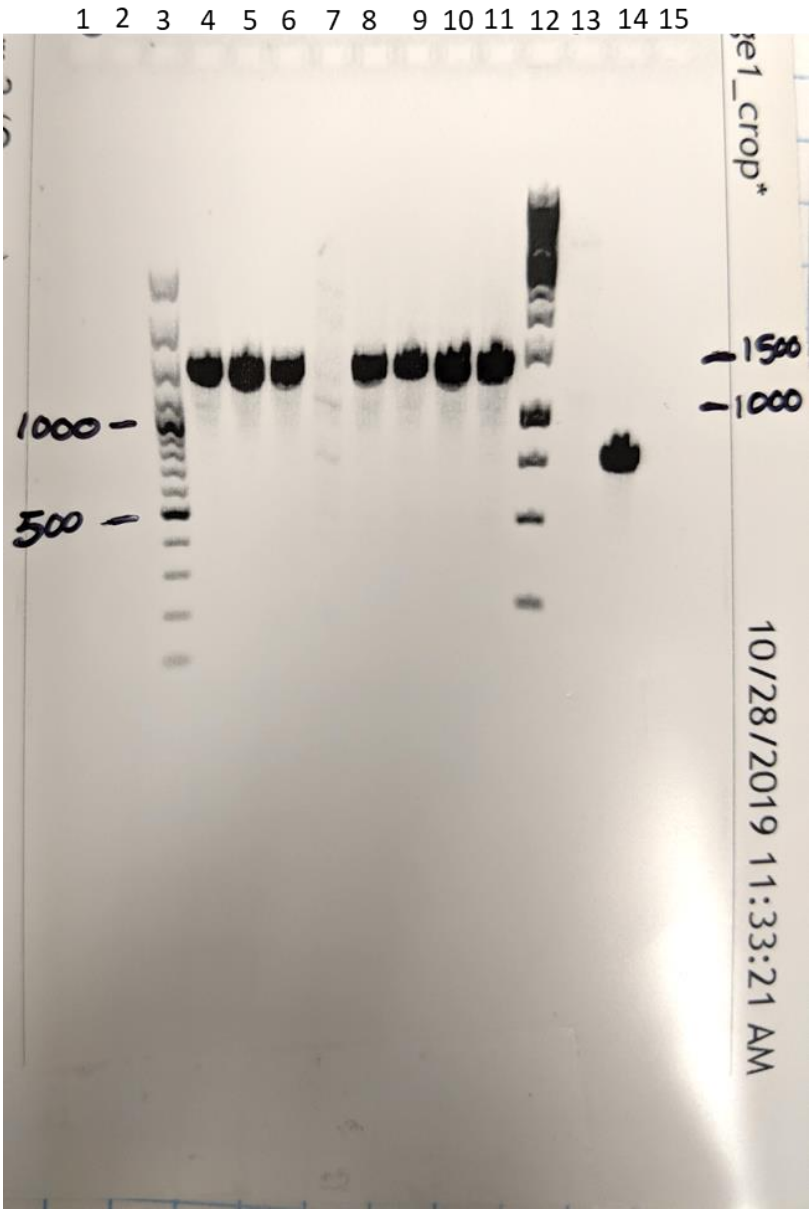

Fig. 3.8. PCR screen results for a 1392-bp portion of the Hygromycin resistance cassette. Products were run on a 1.0% agarose gel to evaluate evidence of "full" (1392/1443 bp) Hygromycin resistance gene cassette integration into *S. musiva* isolate MN-14 genomic DNA by directed disruption. Lane Identity: 1 = Negative Control, 2 = Empty, 3 = 100bp plus Gene Ruler (Ready-to-Use), 4-11 = Putative Directed Disruption Transformants, 12 = 1kb Ready-to-Use Ladder, 13 = WT MN-14 (Non-Transformed), 14 = Random Insertion Partial Gene Positive Control, 15 = EMPTY.

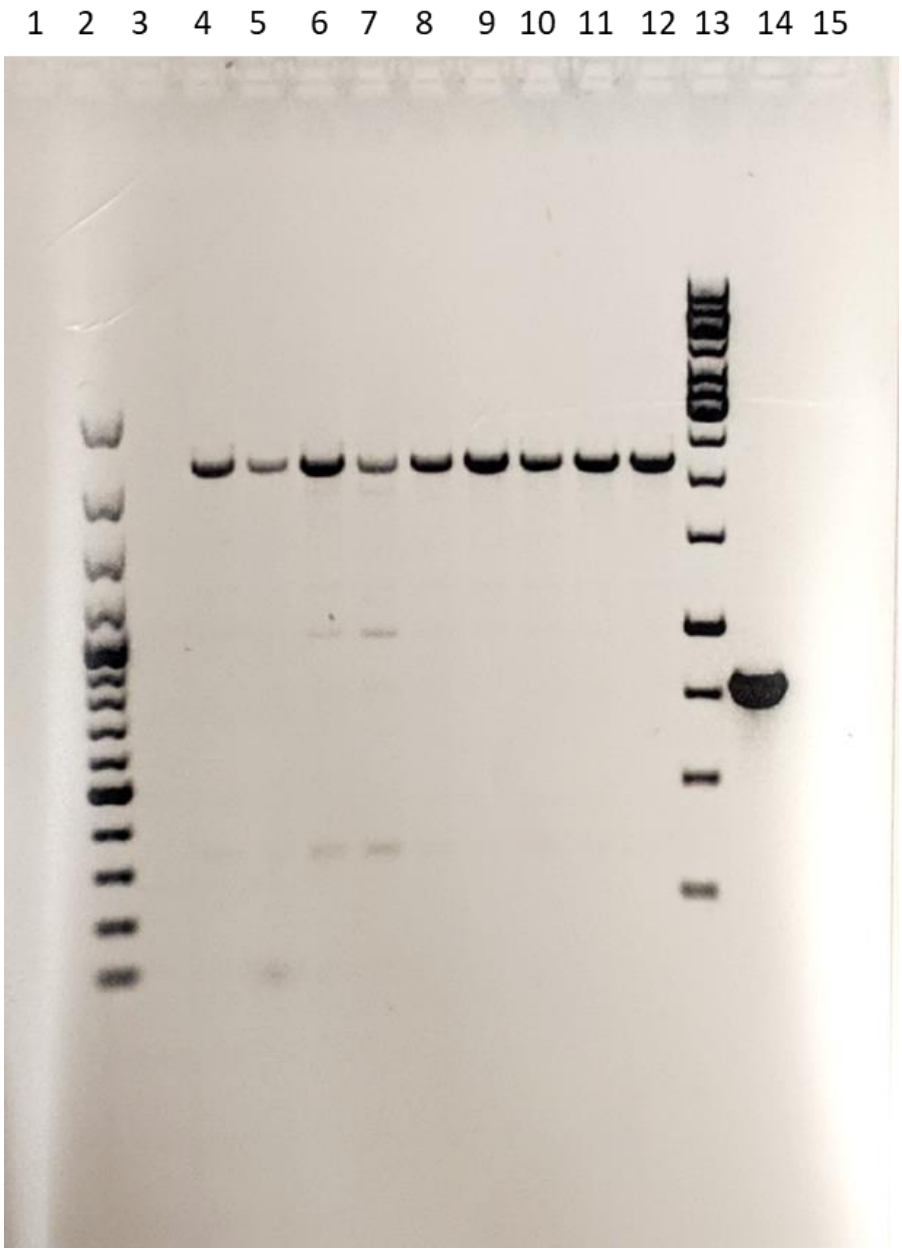

Fig. 3.9. PCR screen results for a 2136-bp PCR product indicating failed disruption of the WT MN-14 *nrps1* gene by ATMT. Products were run on a 1.0% agarose gel to evaluate evidence of failed *nrps1* disruption. Lane Identity: 1 = Negative Control, 2 = 100bp plus Gene Ruler (Ready-to-Use), 3 = EMPTY, 4-11 = Putative Directed Disruption Transformants, 12 = WT MN-14 (Non-Transformed), 13 = 1kb Ready-to-Use Ladder, 14 = Random Insertion Partial Gene Positive Control, 15 = EMPTY.

Table 3.1. Primers used to screen putative random insertion and directed disruption transformants of *S. musiva* isolate MN-14.

Primer Name	Primer Sequence (5' → 3')
INTEG_FOR3	GCCTGACCTATTGCATCTC
pPGW_HygR_REV1	GTCGGCATCTACTCTATTCC
pPGW_HygR_FOR1	TATTGAAGGAGCATTTTGGG
NRPS1_WT_FOR2	TGATTATACAGGAAGGCTTCAG
NRPS1_WT_REV3	CCTTCTCCATAATATTCGAGCT

3.5. Literature cited

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Chapter 4: General Discussion and Conclusion

4.1. Summary

Populus hybrids are economically and ecologically important due to their presence in native populations, their utilization in shelterbelts, and their role in supplying raw material for fiber and biofuel (Bier 1939; Ostry and McNabb 1985; Krupinksy 1989). However, the full potential of *Populus* species may not be realized until concerns regarding disease management are sufficiently addressed (Weiland et al. 2005; LeBoldus et al. 2009; Ostry et al. 2014; Dunnell et al. 2016). *Sphaerulina musiva* Peck., a fungal pathogen native to the north-eastern United States, is the most severe of the diseases of hybrid poplar (Newcombe and Ostry 2001). *Sphaerulina musiva* causes leaf spot on its sympatric host, *P. deltoides* (Feau et al. 2010; Dunnell and LeBoldus 2016). On allopatric species, such as *P. trichocarpa*, and exotic *Populus* hybrids, both leaf spot and necrotic lesions or stem cankers are observed (Bier 1939; Newcombe and Ostry 2001; Dunnell and LeBoldus 2016). In extreme cases, stem cankers may coalesce and girdle the tree, causing loss of structural integrity and a predisposition to wind breakage (Schreiner 1972; Ostry and McNabb 1983; Ostry 1987).

Chemical, biological, and cultural control are largely inadequate for controlling *S. musiva*, due to the high cost of multiple applications over a rotation or poor efficacy

(Carbon 1972; McNabb et al. 1982; Ostry 1987; Gyenis et al. 2003). As such, the development of resistant breeding stock is currently the only viable option for the management of Septoria stem canker (Newcombe and Ostry 2001; Muchero et al. 2018). Classic plant pathology approaches have been successful in providing qualitative evidence of recessive resistance (Newcombe and Ostry 2001). Genomic approaches utilize powerful resources, such as the well-established reference of *P. trichocarpa*, to expedite the identification of host genes related to Septoria stem canker by QTL mapping, genome-wise association studies, and RNA-seq (Tuskan et al. 2006; Wullschleger et al. 2013; Muchero et al. 2018; Abraham et al. 2019; Simon et al., unpublished). The current host-pathogen paradigm was first described by Jones and Dangl (2006) in their Zig-Zag model characterizing the evolutionary arms race between host and pathogen.

The first study described herein characterizes the genetic contribution of the host with respect to Septoria stem canker resistance. QTL were identified using a genetic map in conjunction with Septoria stem canker phenotypes to reveal a correlation between a T × D F_2 pseudo-backcross population of 230 genotypes and a marker on Linkage Group 16 at 74.56 cM. Genes within a 1,000-Kb window centered on the significant marker were filtered based on three parameters: 1) evidence of positive selection (Ka/Ks > 1); 2) annotations related to plant immunity; and 3) the published literature. Interestingly, a field study conducted on progeny from the 52-124 family

infected with Septoria stem canker also found a QTL on Linkage Group 16, although the QTL reported was approximately 3,000,000 bp away from the QTL reported here (Simon et al., unpublished). It is likely that both studies have identified a shared association between Septoria stem canker and a gene(s) located somewhere on Linkage Group 16.

Within the QTL described above, one orthologous gene pair (Potri.016G122700 and Podel.16G128900) had an annotation of *Concanavalin A-like lectin protein kinase* and evidence of positive selection (Ka/Ks ~ 3.14). Lectin-receptor proteins are responsive to both abiotic and biotic stress (Joshi et al. 2010; Ye et al. 2017). Additionally, the jack bean lectin, Concanavalin A, binds to mannans on the surface of yeast cells, lyses oomycete zoospores at micromolar concentrations, and disrupts the adherence of *Magnaporthe grisea* conidia to surfaces such as Teflon by interacting with components of the extracellular matrix secreted at the apex of conidia (Olson and Liener 1967; Sing and Bartnicki-Garcia 1975; Byrt et al. 1982; Hamer et al. 1988; Goldstein and Poretz 2012). Additionally, two poplar lectins were identified by Muchero *et al.* (2018) as mediating the interaction between *S. musiva* and pure *P. trichocarpa*: 1) a *L-type lectin receptor-like protein kinase* associated with resistance, and 2) a *G-type lectin receptor-like protein kinase* associated with susceptibility. The characterization of the orthologous gene pair identified within the list of candidate genes, as well as the other promising candidate genes discussed, will be helpful in understanding the *Populus-Sphaerulina* pathosystem.

The second study in this thesis involved the development of a protocol for the transformation of *S. musiva* and the screening of putative transformants. One method of testing the contribution of certain effectors to disease development is to disrupt or knockout the effector and evaluate the subsequent change in phenotype. Studies taking advantage of the ability of *Agrobacterium tumefaciens* bacteria to transform fungal pathogens through homologous recombination-mediated disruption have been widely reported (Dobinson et al. 2004; Foster et al. 2014; Wang et al. 2016; Abraham 2019). However, previous, in-house attempts at utilizing *Agrobacterium tumefaciens*-mediated transformation to transform *S. musiva* have been unsuccessful. Although this study lacked the scientific rigor necessary for hypothesis testing, it appeared to be capable of producing *S. musiva* transformants using *A. tumefaciens*-mediated transformation (ATMT). Off-site transformation events in attempted site-directed disruption were apparent, based upon PCR screening. Further work needs to be done to develop a trustworthy, reliable protocol for the disruption of genes by ATMT. However, these concerns are likely to be addressed with better experimental planning and could lead to gene-disrupted transformation of *S. musiva*.

4.2. Implications for management

The genome of *P. trichocarpa* was estimated to be approximately 485,000,000 base pairs and contain more than 45,000 protein-coding genes (Tuskan et al. 2006).

Compared to *Arabidopsis*, the *Populus* genome has a large repertoire of genes with defense-related domains such as leucine-rich repeats (ca. 1,271) and NB-ARC domains (ca. 302; Tuskan et al. 2006). Efforts, therefore, must be taken to reduce the number of candidate genes involved in controlling *Populus*-*Sphaerulina* interactions. Although more evidence must be acquired to determine which genes close to the QTL on Linkage Group 16 may be important in mediating the *Sphaerulina*-*Populus* interaction, breeding programs may benefit from screening parents and progeny for this QTL. Determining the effect of this allele on the resistant and susceptible response to Septoria stem canker in pure species and hybrids with different genetic backgrounds would also be helpful. Similarly, the characterization of *S. musiva* effectors with ATMT disruption techniques may aid in the identification of complementary host *R* genes (Flor 1977). As such, the development of an operational protocol for the generation of gene-directed disruption transformants of *S. musiva* will provide a new tool with which to experimentally test hypotheses of effector function and targets.

4.3. Future research

The work presented in this thesis provides a foundation upon which more research may be conducted. Aside from the recommendations to improve the ATMT protocol outlined above, new questions may be developed from both studies. Further research on both host and pathogen genetic contributions to the resistant and susceptible host response

in the *Sphaerulina-Populus* pathosystem is necessary to lead to practical management of Septoria stem canker.

A candidate gene table was filtered to identify genes centered on a significant marker which may be modulating disease resistance in the population of TD × D hybrids studied. However, the characterization of the role of these genes in host-pathogen interactions must be further addressed. Differential gene expression levels, as quantified by experiments such as RNA-seq or qPCR, are suitable metrics upon which candidate gene lists may be further narrowed. In addition, resolving the inconsistency between the locations of the significant QTL on Linkage Group 16 between field and greenhouse data is necessary for breeding programs to screen genotypes. Studying the effect that Concanavalin A has on conidial attachment to *Populus* stems may prove interesting, especially if evidence can be obtained that the extracellular matrix of *S. musiva* conidia binds to ConA.

Previous RNAseq experiments detected putative effector proteins in the MN-14 isolate of *S. musiva* which may play a role in suppressing the immune response of susceptible *Populus* hosts to pathogen-associated molecular patterns (PAMPs) or other signs of infection (Dunnell 2016). The ATMT protocol described, although needing many modifications prior to being used for hypothesis testing, may serve an important role in studying the effect of putative *S. musiva* effector genes on Septoria stem canker. If effector-knockout transformants of *S. musiva* can be generated, it may be possible to

compare the stem canker phenotype from inoculation by an effector-knockout transformant of *S. musiva* to WT *S. musiva* and identify loci in *Populus* species which may interact with the disrupted effector. Associated host proteins identified this way may then be used for traditional breeding program selection. Alternatively, cloning may be used to modify otherwise susceptible poplar stock to be resistant.

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Appendix

Appendix A.1. All (114) orthologous *P. trichocarpa* and *P. deltoides* genes collected from the 1,000 kb window centered on marker scaffold_16_12428393 (marker position = 74.56263779 cM on Linkage Group 16). BLASTP %Identity have been rounded to 2 decimal places; Ka, Ks, and Ka/Ks have been rounded to 2 decimal places.

***P. trichocarpa* (v3.0) Phytozome Gene Model**	***P. deltoides* (WV94 v2.1) Phytozome Gene Model**	**BLASTP %Identity**	**BLASTP E-Score**	***P. trichocarpa* Gene Annotation**	***P. deltoides* Gene Annotation**	**Ka**	**Ks**	**Ka/Ks**
Potri.016G119000	Podel.16G124300	93.96	1E-101	AGAMOUS-like 62	AGAMOUS-like 62	ND	ND	ND
Potri.016G126600	Podel.16G132000	99.05	1E-68	Tautomerase/MIF superfamily protein	Tautomerase/MIF superfamily protein	ND	ND	ND
Potri.016G126700	Podel.16G132100	100	6E-80	Tautomerase/MIF superfamily protein	Tautomerase/MIF superfamily protein	ND	ND	ND
Potri.016G117700	Podel.16G123200	95.24	7E-66	transducin family protein / WD-40 repeat family protein	transducin family protein / WD-40 repeat family protein	0.021	0.000	INF
Potri.016G120800	Podel.16G126400	98.17	3E-117	APRATAXIN-like	APRATAXIN-like	0.008	0.000	INF
Potri.016G121600	Podel.16G127600	92.98	2E-32	ND	ND	0.129	0.027	4.696
Potri.016G122700	Podel.16G128900	88.24	3E-81	Concanavalin A-like lectin protein kinase family protein	lectin receptor kinase a4.1	0.376	0.120	3.142
Potri.016G125800	Podel.16G131100	90.91	9E-39	ND	BRCA2-like B	0.075	0.032	2.310
Potri.016G128000	Podel.16G133700	94.31	1E-71	Ribosomal protein S27a / Ubiquitin family protein	Ribosomal protein S27a / Ubiquitin family protein	0.025	0.012	2.086
Potri.016G117400	Podel.16G122900	96.51	0	ALC-interacting protein 1	ALC-interacting protein 1	0.015	0.009	1.691
Potri.016G124000	Podel.16G129400	96.34	5E-96	ND	ND	0.016	0.009	1.679
Potri.016G127400	Podel.16G133000	96.55	2E-58	ND	ND	0.155	0.104	1.490
Potri.016G126800	Podel.16G132200	93.97	4E-71	Tautomerase/MIF superfamily protein	Tautomerase/MIF superfamily protein	1.301	0.894	1.457
Potri.016G124400	Podel.16G129800	88.89	1E-161	UDP-glucosyl transferase 85A3	UDP-glucosyl transferase 85A3	0.017	0.012	1.443
Potri.016G120300	Podel.16G125700	89.09	1E-64	Plant protein of unknown function (DUF247)	Plant protein of unknown function (DUF247)	1.254	0.874	1.434
Potri.016G129800	Podel.16G135500	68.42	3E-28	iron-sulfur cluster binding;electron carriers;4 iron, 4 sulfur cluster binding	iron-sulfur cluster binding;electron carriers;4 iron, 4 sulfur cluster binding	0.204	0.150	1.363
Potri.016G122200	Podel.16G128200	97.05	0	gamma interferon responsive lysosomal thiol (GILT) reductase family protein	Thioredoxin superfamily protein	0.013	0.011	1.215

Potri.016G129400	Podel.16G135200	97.45	4E-81	hydroxyproline-rich glycoprotein family protein	hydroxyproline-rich glycoprotein family protein	0.026	0.024	1.115
Potri.016G128100	Podel.16G134000	96.86	7E-177	methyltransferases	methyltransferases	0.098	0.089	1.107
Potri.016G122100	Podel.16G128100	94.59	3E-43	ND	ND	0.037	0.034	1.078
Potri.016G120500	Podel.16G126200	84.89	0	receptor like protein 6	receptor like protein 27	0.122	0.125	0.975
Potri.016G121300	Podel.16G127300	88.16	2E-40	ammonium transporter 2	ammonium transporter 2	0.055	0.057	0.965
Potri.016G125100	Podel.16G130400	90.14	7E-82	Ribosomal protein S4 (RPS4A) family protein	Ribosomal protein S4 (RPS4A) family protein	0.036	0.040	0.901
Potri.016G123300	Podel.16G128600	91.51	0	lectin receptor kinase a4.1	Concanavalin A-like lectin protein kinase family protein	0.040	0.044	0.898
Potri.016G118600	Podel.16G124100	97.65	1E-51	electron transport SCO1/SenC family protein	electron transport SCO1/SenC family protein	0.019	0.021	0.877
Potri.016G124500	Podel.16G129900	88	1E-39	UDP-glucosyl transferase 85A3	UDP-glucosyl transferase 85A3	0.321	0.390	0.823
Potri.016G126000	Podel.16G131400	90.83	0	MATE efflux family protein	MATE efflux family protein	0.033	0.041	0.798
Potri.016G129200	Podel.16G135100	65.65	1E-49	ND	ND	0.088	0.112	0.784
Potri.016G120600	Podel.16G126200	94.39	0	receptor like protein 6	receptor like protein 27	0.025	0.032	0.767
Potri.016G123500	Podel.16G128800	86.64	0	lectin receptor kinase a4.1	lectin receptor kinase a4.1	0.059	0.089	0.660
Potri.016G118900	Podel.16G124300	95.19	1E-131	AGAMOUS-like 62	AGAMOUS-like 62	0.042	0.066	0.641
Potri.016G123400	Podel.16G128500	91.01	3E-122	RGA-like 2	RGA-like 2	0.037	0.060	0.627
Potri.016G118200	Podel.16G123600	90.28	2E-36	ND	ND	0.047	0.075	0.627
Potri.016G118100	Podel.16G123500	91.38	5E-34	SAC domain-containing protein 8	SAC domain-containing protein 8	0.072	0.118	0.609
Potri.016G125700	Podel.16G131000	95.73	1E-114	Brassinosteroid signalling positive regulator (BZR1) family protein	Brassinosteroid signalling positive regulator (BZR1) family protein	0.022	0.036	0.592
Potri.016G122400	Podel.16G128300	97.2	4E-67	ND	ND	0.013	0.022	0.587
Potri.016G119700	Podel.16G125100	97.28	0	metallopeptidase M24 family protein	metallopeptidase M24 family protein	0.013	0.022	0.586
Potri.016G122300	Podel.16G128300	98.63	2E-47	ND	ND	0.007	0.013	0.565
Potri.016G121800	Podel.16G127800	98.42	0	myb-like HTH transcriptional regulator family protein	myb-like HTH transcriptional regulator family protein	0.007	0.012	0.560
Potri.016G126100	Podel.16G131500	95.36	0	Integrase-type DNA-binding superfamily protein	Integrase-type DNA-binding superfamily protein	0.009	0.017	0.557
Potri.016G121200	Podel.16G127200	96.08	6E-164	BCL-2-associated athanogene 4	BCL-2-associated athanogene 4	0.017	0.031	0.550

Potri.016G127800	Podel.16G133500	97.62	0	cation/H+ exchanger 26	cation/H+ exchanger 26	0.011	0.021	0.540
Potri.016G130000	Podel.16G135700	97.95	0	UDP-Glycosyltransferase superfamily protein	UDP-Glycosyltransferase superfamily protein	0.038	0.071	0.530
Potri.016G119500	Podel.16G124800	98.98	0	Mitochondrial substrate carrier family protein	Mitochondrial substrate carrier family protein	0.035	0.067	0.528
Potri.016G127500	Podel.16G133100	97.39	0	ARM repeat superfamily protein	ARM repeat superfamily protein	0.013	0.025	0.512
Potri.016G123200	Podel.16G128500	96.17	6E-129	RGA-like 2	RGA-like 2	0.018	0.037	0.499
Potri.016G128900	Podel.16G134800	98.98	0	Pentatricopeptide repeat (PPR) superfamily protein	Pentatricopeptide repeat (PPR) superfamily protein	0.004	0.009	0.490
Potri.016G129000	Podel.16G134900	95.46	0	SCAR homolog 2	SCAR homolog 2	0.017	0.036	0.483
Potri.016G119600	Podel.16G124900	98.31	0	Tetratricopeptide repeat (TPR)-like superfamily protein	Tetratricopeptide repeat (TPR)-like superfamily protein	0.008	0.016	0.475
Potri.016G127900	Podel.16G133600	98.12	0	Protein phosphatase 2C family protein	Protein phosphatase 2C family protein	0.008	0.019	0.431
Potri.016G127600	Podel.16G133200	98.63	0	ND	ND	0.006	0.014	0.428
Potri.016G119900	Podel.16G125300	94.86	0	ARM repeat superfamily protein	ARM repeat superfamily protein	0.007	0.016	0.420
Potri.016G122500	Podel.16G128400	96.83	0	RGA-like 2	RGA-like 2	0.014	0.033	0.416
Potri.016G124700	Podel.16G130100	99.16	9E-174	ferritin 4	ferretin 1	0.037	0.088	0.415
Potri.016G124600	Podel.16G130000	98.35	5E-84	UDP-glucosyl transferase 85A5	UDP-glucosyl transferase 85A5	0.012	0.030	0.393
Potri.016G125500	Podel.16G130800	97.08	0	TRICHOME BIREFRINGENCE-LIKE 34	TRICHOME BIREFRINGENCE-LIKE 34	0.012	0.031	0.378
Potri.016G121500	Podel.16G127500	97.12	0	nudix hydrolase homolog 15	nudix hydrolase homolog 15	0.012	0.034	0.360
Potri.016G120900	Podel.16G126500	98.64	0	diaminopimelate epimerase family protein	diaminopimelate epimerase family protein	0.006	0.018	0.329
Potri.016G120000	Podel.16G125400	96.71	0	DNAJ heat shock N-terminal domain-containing protein	DNAJ heat shock N-terminal domain-containing protein	0.015	0.047	0.326
Potri.016G128200	Podel.16G134100	99.49	4E-141	Uncharacterised protein family (UPF0497)	Uncharacterised protein family (UPF0497)	0.002	0.007	0.310
Potri.016G121000	Podel.16G126600	98.8	1E-116	PEBP (phosphatidylethanolamine-binding protein) family protein	PEBP (phosphatidylethanolamine-binding protein) family protein	0.005	0.018	0.294
Potri.016G122000	Podel.16G126900	99.56	3E-169	Thioredoxin superfamily protein	Thioredoxin superfamily protein	0.002	0.007	0.293
Potri.016G128800	Podel.16G134700	97.67	1E-121	ND	ND	0.010	0.035	0.293
Potri.016G129600	Podel.16G135300	99.38	3E-118	Nuclear transport factor 2 (NTF2) family protein	Nuclear transport factor 2 (NTF2) family protein	0.003	0.010	0.269

Potri.016G125600	Podel.16G130900	99.78	0	TRICHOME BIREFRINGENCE-LIKE 35	TRICHOME BIREFRINGENCE-LIKE 35	0.001	0.004	0.268
Potri.016G128400	Podel.16G134300	98.8	0	RNI-like superfamily protein	RNI-like superfamily protein	0.006	0.022	0.268
Potri.016G117800	Podel.16G123300	98.93	0	transducin family protein / WD-40 repeat family protein	transducin family protein / WD-40 repeat family protein	0.004	0.017	0.252
Potri.016G123900	Podel.16G129300	94.77	0	ND	ND	0.007	0.027	0.252
Potri.016G129500	Podel.16G135300	84.31	4E-96	Nuclear transport factor 2 (NTF2) family protein	Nuclear transport factor 2 (NTF2) family protein	0.082	0.327	0.251
Potri.016G121900	Podel.16G126800	98.35	2E-180	gamma interferon responsive lysosomal thiol (GILT) reductase family protein	gamma interferon responsive lysosomal thiol (GILT) reductase family protein	0.007	0.031	0.231
Potri.016G126500	Podel.16G132000	90.98	2E-77	Tautomerase/MIF superfamily protein	Tautomerase/MIF superfamily protein	0.045	0.196	0.228
Potri.016G120200	Podel.16G125600	97.71	0	APRATAXIN-like	APRATAXIN-like	0.010	0.044	0.227
Potri.016G117500	Podel.16G123000	93.1	0	fatty acid desaturase 8	fatty acid desaturase 8	0.008	0.036	0.216
Potri.016G129700	Podel.16G135400	98.98	0	PLAC8 family protein	PLAC8 family protein	0.004	0.021	0.210
Potri.016G119400	Podel.16G124700	97.38	0	UbiA prenyltransferase family protein	UbiA prenyltransferase family protein	0.004	0.017	0.206
Potri.016G121400	Podel.16G127400	98.97	0	ammonium transporter 2	ammonium transporter 2	0.005	0.023	0.203
Potri.016G118400	Podel.16G123800	99.13	0	Transducin family protein / WD-40 repeat family protein	Transducin family protein / WD-40 repeat family protein	0.004	0.019	0.203
Potri.016G126300	Podel.16G131700	98.79	0	inflorescence meristem receptor-like kinase 2	inflorescence meristem receptor-like kinase 2	0.006	0.029	0.203
Potri.016G128600	Podel.16G134500	98.65	0	iron regulated 2	iron regulated 2	0.006	0.030	0.200
Potri.016G119200	Podel.16G124500	98.12	6E-157	CAX-interacting protein 2	CAX-interacting protein 2	0.009	0.045	0.195
Potri.016G123800	Podel.16G129200	98.86	0	tetratricopeptide repeat (TPR)-containing protein	tetratricopeptide repeat (TPR)-containing protein	0.004	0.021	0.190
Potri.016G129100	Podel.16G135000	98.37	0	NAD(P)-binding Rossmann-fold superfamily protein	NAD(P)-binding Rossmann-fold superfamily protein	0.006	0.032	0.183
Potri.016G126400	Podel.16G131800	98.22	3E-156	prenylated RAB acceptor 1.B4	prenylated RAB acceptor 1.B4	0.008	0.045	0.182
Potri.016G118300	Podel.16G123700	95.22	0	SAC domain-containing protein 8	SAC domain-containing protein 8	0.005	0.028	0.179
Potri.016G128500	Podel.16G134400	98.44	0	iron regulated 2	iron regulated 2	0.007	0.041	0.167
Potri.016G119800	Podel.16G125200	98.73	4E-166	RAN binding protein 1	RAN binding protein 1	0.005	0.034	0.162
Potri.016G125200	Podel.16G130500	98.79	0	Mitochondrial substrate carrier family protein	Mitochondrial substrate carrier family protein	0.005	0.034	0.156
Potri.016G117300	Podel.16G122800	99.29	0	Homeodomain-like superfamily protein	Homeodomain-like superfamily protein	0.003	0.020	0.150

Potri.016G125000	Podel.16G130300	98.46	0	Peroxidase superfamily protein	Peroxidase superfamily protein	0.007	0.047	0.147
Potri.016G123600	Podel.16G129000	99.2	0	ARM repeat superfamily protein	ARM repeat superfamily protein	0.003	0.024	0.147
Potri.016G124900	Podel.16G130200	95.29	8E-176	ferritin 4	ferretin 1	0.009	0.062	0.142
Potri.016G119300	Podel.16G124600	99.53	0	AMP deaminase, putative / myoadenylate deaminase, putative	AMP deaminase, putative / myoadenylate deaminase, putative	0.002	0.015	0.131
Potri.016G118700	Podel.16G124100	99.09	0	electron transport SCO1/SenC family protein	electron transport SCO1/SenC family protein	0.004	0.031	0.130
Potri.016G124300	Podel.16G129700	98.86	4E-60	ND	ND	0.005	0.038	0.129
Potri.016G118500	Podel.16G123900	97.29	1E-146	glutathione S-transferase tau 7	glutathione S-transferase tau 7	0.012	0.094	0.126
Potri.016G120700	Podel.16G126300	96.27	0	Major facilitator superfamily protein	Major facilitator superfamily protein	0.019	0.152	0.123
Potri.016G121100	Podel.16G126700	92.32	0	MIF4G domain-containing protein / MA3 domain-containing protein	MIF4G domain-containing protein / MA3 domain-containing protein	0.005	0.048	0.111
Potri.016G124200	Podel.16G129600	97.33	5E-48	ND	ND	0.012	0.107	0.110
Potri.016G127700	Podel.16G133300	99.06	0	SEC14 cytosolic factor family protein / phosphoglyceride transfer family protein	SEC14 cytosolic factor family protein / phosphoglyceride transfer family protein	0.004	0.039	0.105
Potri.016G121700	Podel.16G127700	99.43	6E-127	Basic-leucine zipper (bZIP) transcription factor family protein	Basic-leucine zipper (bZIP) transcription factor family protein	0.002	0.025	0.098
Potri.016G129900	Podel.16G135600	99.31	0	alpha-soluble NSF attachment protein 2	alpha-soluble NSF attachment protein 2	0.003	0.032	0.093
Potri.016G123700	Podel.16G129100	95.83	2E-179	Protein of unknown function (DUF688)	Protein of unknown function (DUF688)	0.005	0.053	0.086
Potri.016G118000	Podel.16G123400	98.86	0	acyl-CoA oxidase 4	acyl-CoA oxidase 4	0.004	0.054	0.073
Potri.016G128300	Podel.16G134200	99.66	0	WRKY DNA-binding protein 33	WRKY DNA-binding protein 33	0.001	0.021	0.071
Potri.016G125300	Podel.16G130600	98.83	3E-122	Protein of unknown function, DUF538	Protein of unknown function, DUF538	0.005	0.081	0.063
Potri.016G119100	Podel.16G124400	99.53	0	Plant protein of unknown function (DUF828)	Plant protein of unknown function (DUF828)	0.002	0.033	0.061
Potri.016G124100	Podel.16G129500	99.62	0	Ribosomal protein S4 (RPS4A) family protein	Ribosomal protein S4 (RPS4A) family protein	0.002	0.033	0.051
Potri.016G120100	Podel.16G125500	99.83	0	Thiamine pyrophosphate dependent pyruvate decarboxylase family protein	Thiamine pyrophosphate dependent pyruvate decarboxylase family protein	0.001	0.016	0.045
Potri.016G118800	Podel.16G124200	100	4E-47	ND	ND	0.000	0.022	0.000
Potri.016G125400	Podel.16G130700	100	4E-164	PYR1-like 4	PYR1-like 4	0.000	0.031	0.000
Potri.016G126200	Podel.16G131600	100	0	ND	ND	0.000	0.010	0.000
Potri.016G127200	Podel.16G132800	100	5E-89	ND	ND	0.000	0.000	0.000

Potri.016G127300	Podel.16G132900	100	2E-78	Tautomerase/MIF superfamily protein	Tautomerase/MIF superfamily protein	0.000	0.012	0.000
Potri.016G128700	Podel.16G134600	100	1E-104	ND	ND	0.000	0.011	0.000

Appendix A.2. All (23) *P. deltoides* genes collected from the 1,000 kb window centered on marker scaffold_16_12428393 (marker position = 74.56263779 cM on Linkage Group 16) without a syntenic *P. trichocarpa* orthologue.

***P. deltoides* (WV94 v2.1) Phytozome Gene Model Number**	***P. deltoides* Gene Annotation**
Podel.16G123100	SELT-like protein precursor
Podel.16G124000	glutathione S-transferase tau 7
Podel.16G125000	Mitochondrial substrate carrier family protein
Podel.16G125800	receptor like protein 7
Podel.16G125900	receptor like protein 33
Podel.16G126000	ND
Podel.16G126100	receptor like protein 19
Podel.16G127000	MIF4G domain-containing protein / MA3 domain-containing protein
Podel.16G127100	MIF4G domain-containing protein / MA3 domain-containing protein
Podel.16G127900	gamma interferon responsive lysosomal thiol (GILT) reductase family protein
Podel.16G128000	Thioredoxin superfamily protein
Podel.16G128700	RGA-like protein 3
Podel.16G131200	BRCA2-like B
Podel.16G131300	ND
Podel.16G131900	Tautomerase/MIF superfamily protein
Podel.16G132300	receptor like protein 33
Podel.16G132400	Tautomerase/MIF superfamily protein
Podel.16G132500	receptor like protein 6
Podel.16G132600	ND
Podel.16G132700	ND
Podel.16G133400	Polynucleotidyl transferase, ribonuclease H-like superfamily protein
Podel.16G133800	Ribosomal protein S27a / Ubiquitin family protein
Podel.16G133900	methyltransferases

Appendix A.3. All (14) *P. trichocarpa* genes collected from the 1,000 kb window centered on marker scaffold_16_12428393 (marker position = 74.56263779 cM on Linkage Group 16) without a syntenic *P. deltoides* orthologue.

***P. trichocarpa* (v3.0) Phytozome Gene Model Number**	***P. trichocarpa* Gene Annotation**
Potri.016G117600	ND
Potri.016G117900	receptor like protein 33
Potri.016G120400	receptor like protein 6
Potri.016G122600	receptor like protein 6
Potri.016G122800	BREAST CANCER 2 like 2A
Potri.016G122900	UDP-Glycosyltransferase superfamily protein
Potri.016G123000	ND
Potri.016G123100	multidrug resistance-associated protein 14
Potri.016G124800	ND
Potri.016G125900	multidrug resistance-associated protein 14
Potri.016G126900	ND
Potri.016G127000	Protein kinase family protein with ARM repeat domain
Potri.016G127100	ND
Potri.016G129300	SELT-like protein precursor

Appendix A.4. All (15) orthologous *P. trichocarpa* and *P. deltoides* genes collected from the 1,000 kb window centered on marker scaffold_16_12428393 (marker position = 74.56263779 cM on Linkage Group 16) for which (Ka/Ks) > 1.

P. trichocarpa **(v3.0) Phytozome Gene Model**	***P. deltoides*** **(WV94 v2.1) Phytozome Gene Model**	**BLASTP %Identity**	**BLASTP E-Score**	***P. trichocarpa*** **Gene Annotation**	***P. deltoides*** **Gene Annotation**	**Ka**	**Ks**	**Ka/Ks**
Potri.016G121600	Podel.16G127600	92.98	2E-32	ND	ND	0.129	0.027	4.696
Potri.016G122700	Podel.16G128900	88.24	3E-81	Concanavalin A-like lectin protein kinase family protein	lectin receptor kinase a4.1	0.376	0.120	3.142
Potri.016G125800	Podel.16G131100	90.91	9E-39	ND	BRCA2-like B	0.075	0.032	2.310
Potri.016G128000	Podel.16G133700	94.31	1E-71	Ribosomal protein S27a / Ubiquitin family protein	Ribosomal protein S27a / Ubiquitin family protein	0.025	0.012	2.086
Potri.016G117400	Podel.16G122900	96.51	0	ALC-interacting protein 1	ALC-interacting protein 1	0.015	0.009	1.691
Potri.016G124000	Podel.16G129400	96.34	5E-96	ND	ND	0.016	0.009	1.679
Potri.016G127400	Podel.16G133000	96.55	2E-58	ND	ND	0.155	0.104	1.490
Potri.016G126800	Podel.16G132200	93.97	4E-71	Tautomerase/MIF superfamily protein	Tautomerase/MIF superfamily protein	1.301	0.894	1.457
Potri.016G124400	Podel.16G129800	88.89	1E-161	UDP-glucosyl transferase 85A3	UDP-glucosyl transferase 85A3	0.017	0.012	1.443
Potri.016G120300	Podel.16G125700	89.09	1E-64	Plant protein of unknown function (DUF247)	Plant protein of unknown function (DUF247)	1.254	0.874	1.434
Potri.016G129800	Podel.16G135500	68.42	3E-28	iron-sulfur cluster binding;electron carriers;4 iron, 4 sulfur cluster binding	iron-sulfur cluster binding;electron carriers;4 iron, 4 sulfur cluster binding	0.204	0.150	1.363
Potri.016G122200	Podel.16G128200	97.05	0	gamma interferon responsive lysosomal thiol (GILT) reductase family protein	Thioredoxin superfamily protein	0.013	0.011	1.215
Potri.016G129400	Podel.16G135200	97.45	4E-81	hydroxyproline-rich glycoprotein family protein	hydroxyproline-rich glycoprotein family protein	0.026	0.024	1.115
Potri.016G128100	Podel.16G134000	96.86	7E-177	methyltransferases	methyltransferases	0.098	0.089	1.107