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Identification And Characterization Of A Putative Populus E3 RING-H2 Ubiquitin Ligase That When Over-Expressed Is Correlated With Insect Resistance To Orgyia Leucostigma

Justin Burum

IDENTIFICATION AND CHARACTERIZATION OF A PUTATIVE *POPULUS* E3 RING-H2 UBIQUITIN LIGASE THAT WHEN OVER-EXPRESSED IS CORRELATED WITH INSECT RESISTANCE TO *ORGYIA LEUCOSTIGMA*

by

Justin Burum
Bachelor of Arts, Gustavus Adolphus College, 2006
Bachelor of Science, Minnesota State University Mankato, 2008

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This dissertation, submitted by Justin Dylan Burum in partial fulfillment of the requirements for the degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisor Committee under whom the work has been done and is hereby approved.

Dr. Steven Ralph, Chairperson

Dr. Turk Rhen

Dr. William Sheridan

Dr. Peter Meberg

Dr. Roxanne Vaughan

This dissertation is being submitted by the appointed advisory committee as having met all of the requirements of the School of Graduate Studies at the University of North Dakota and is hereby approved.

Wayne Swisher
Dean of the School of Graduate Studies

April 29, 2016
Date

PERMISSION

Title Identification and Characterization of a Putative *Populus* E3 RING-H2 Ubiquitin Ligase that when Over-Expressed is Correlated with Insect Resistance to *Orgyia Leucostigma*

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Justin Burum
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ABSTRACT

Insect herbivory is a major stress on plants that results in significant economic losses to forest plantations and natural forests. In response, *Populus* have evolved a suite of constitutive and inducible defenses to deter insect feeding. Despite advances in technologies and the sequencing of the *Populus trichocarpa* genome, defensive pathways against insect herbivores are poorly understood. With the creation of activation tagged (AT) *Populus* we can use a forward genetics approach to identify genes involved in insect resistance (IR).

To explore the genetic architecture of tree defenses, we conducted a forward genetics screen of AT *Populus tremula* x *Populus alba* (*Pt x Pa*) trees using choice bioassays with the purpose of identifying mutants with altered constitutive and induced defenses to *Orgyia leucostigma*. 770 AT mutants from Michigan Technological University were screened using mutant-mutant pairs on unwounded and wounded leaves. Fourteen percent (108 mutants) were identified as candidates for additional choice bioassays against wildtype trees. From this screen we have identified a few candidates that can be investigated with additional bioassays. In addition, we conducted bioassays on nine AT mutants previously identified from a screen at Queen's University to confirm that they had altered IR. One mutant from the QU population, *E8-16*, showed IR during two independent choice bioassays using unwounded leaves. Choice bioassays demonstrated that larvae consumed less area and less weight from *E8-16* compared to wildtype leaf disks. No-choice bioassays, designed to determine if the AT mutant has an

effect on insect weight gain and development, revealed larvae reared on *E8-16* trees gained less wet and dry weight, consumed less leaf material, and delayed larval development.

The next step was to identify where the T-DNA inserted within the *Populus* genome for *E8-16* and which gene(s) were "activated" in response to being in close proximity to cauliflower mosaic virus enhancers on the T-DNA. We used SiteFinding and thermal asymmetric interlaced (TAIL) PCR to map a T-DNA insertion on chromosome 10 for the *E8-16* mutant. Real-time PCR of three genes within 20 kb of the T-DNA revealed *10s12800* had elevated expression in *E8-16* versus *Pt x Pa* wildtype leaves (6.9-fold, nested ANOVA, $p<0.00001$).

To begin to characterize the gene a bioinformatic analysis of the 10s12800 amino acid sequence was conducted which identified a really interesting new gene (RING) domain. 10s12800 is a putative E3 RING-H2 ubiquitin ligase involved in the terminal step of the ubiquitin-proteasome pathway that marks target proteins with ubiquitin for degradation by the 26S proteasome.

Real-time PCR was used to measure absolute abundance of *10s12800* mRNA in a tissue panel consisting of mature leaves, immature leaves, phloem, bark, xylem, petioles, and roots. This analysis revealed *10s12800* is ubiquitously expressed in all tissues with greatest expression in mature leaves. To identify potential defensive pathways influenced by increased *10s12800* expression, we analyzed leaf transcriptomes of *E8-16* and *Pt x Pa* wildtype trees on an Agilent 4x44K *Populus* microarray. Using criteria of $p<0.05$ and a false discovery q value <0.15, only 22 of the 43, 803 gene probes showed expression differences between *Pt x Pa* wildtype and *E8-16*. Among those 22 probes were MADS-

box transcription factors and chitinases. To confirm *10s12800* is responsible for the IR phenotype, *10s12800* full length cDNA was inserted into the pCAMBIA S1300 vector behind a partial superpromoter and introduced into two different *Populus* genetic backgrounds. qPCR screening of transgenic lines revealed one line, *TL4*, with a two-fold increase in expression compared to a vector control (Nested ANOVA, $p<0.05$, $n=5$). Choice assays revealed that *Orgyia leucostigma* larvae preferred to consume vector control trees compared to *TL4* (Nested ANOVA, $p<0.05$, $n=8$ pairs). However, we did not see a statistical difference in insect growth and development in a no-choice assay where larvae were caged on either *TL4* or vector control trees (t-test, $p>0.05$, $n=9$). Collectively, these results suggest that we have identified a putative E3 RING-H2 ubiquitin ligase that may be a regulator of plant defense against *Orgyia leucostigm*a.

CHAPTER I

INTRODUCTION TO DISSERTATION

Thesis Goal and Objectives

Populus species have become an important economic resource for lumber and paper products, carbon sequestration, phytoremediation, and as a potential source for cellulosic ethanol. As such, plantation forestry has become a means to meet the ever-growing demand for these products. Unfortunately, traditional means to protect agricultural crops from insects, such as pesticides and crop rotation, are not applicable when it comes to large plantation forests. Substantial defoliation by insect herbivores can negatively impact forest plantation productivity by slowing plant growth and weakening trees to other opportunistic insects and fungal pathogens that can eventually result in their death. As long-lived sedentary organisms, tree species such as *Populus* have evolved a suite of natural constitutive and inducible defenses to deter or reduce insect herbivory and make the host plant more insect resistant. With the creation of activation tagged (AT) *Populus* populations (Busov et al. 2010; Harrison et al. 2007), we can now use a forward genetics approach to identify genes and gene regulatory networks involved in *Populus* defense to insect herbivores. The purpose of this study was to conduct a forward genetics screen of AT mutants and identify *Populus* genes relevant to insect resistance (IR) to the white-marked tussock moth (WMTM), *Orgyia leucostigma* (J.E. Smith). This thesis

seeks to further advance our understanding of genes involved in IR that could eventually be incorporated into breeding programs to produce superior trees for plantation forestry.

Objective #1: Identify AT *Populus* Mutants With Altered Insect Resistance to *Orgyia leucostigma* Using Choice and No-choice Bioassays.

Populus has become a model system for studying aspects of deciduous tree physiology including wood formation, environmental adaptation, and responses to biotic stresses including pathogens and insect herbivores (Mauriat et al. 2015; Philippe and Bohlman 2007; Jansson and Douglas, 2007; Cronk, 2005; Brunner et al. 2004). The study of plant biology, including plant-insect interactions, has advanced due to the sequencing of the *Populus trichocarpa* genome (Tusken et al. 2006), creation of genetic and physical genome maps (Woolbright et al. 2008; Kelleher et al. 2007; Yin et al. 2004), development of microarray platforms (Ralph et al. 2006; Harding et al. 2005; Brosche et al. 2005; Andersson et al. 2004), large collections of expressed sequence tags and full-length cDNAs (Ralph et al. 2008; Ralph et al. 2006; Sterky et al. 2004; Christopher et al. 2004; Ranjan et al. 2004), RNA-Seq data (Ariani et al. 2015; Liu et al. 2014; Zhang et al. 2014), and proteomic and metabolite studies (Hamanishi et al. 2015; Nilsson et al. 2010; Kieffer et al. 2009). In particular, substantial transcriptomic data following simulated and actual insect herbivory from lepidopteran defoliators has resulted in extensive gene lists that can be used in a reverse genetics approach to identify genes causing IR. However, our understanding of the genes involved in regulating plant defenses is still poorly understood. A literature review discussing *Populus*, their lepidopteran defoliators, and their defenses can be found in chapter 2.

Until the last decade, forward genetics approaches to identify genes involved in plant defense for *Populus* were not possible due to a lack of mutagenized populations. In *Populus*, AT has become an effective insertion mutagenesis approach to developing gain of function mutants (Busov et al. 2010; Harrison et al. 2007). Few forward genetic studies have been conducted on AT plant populations to identify genotypes with altered resistance to insect herbivory (Chen et al. 2012; Ralph 2009). The first screen to identify insect resistant mutants in AT *Populus* was conducted by Ralph (2009) using a population created by Sharon Regan's lab at Queen's University (QU) in Ontario, Canada (Harrison et al. 2007). The purpose of objective #1 of this dissertation was to continue the work started by Ralph (2009) by: 1) conducting additional choice assays with greater biological replication on AT candidates identified from the QU screen, and 2) screening additional AT lines created by Dr. Victor Busov at Michigan Technological University (MTU) in Houghton, MI, using choice bioassays to identify AT mutants with altered IR. Choice bioassays designed to compare feeding preference of WMTM on unwounded leaves were conducted on nine mutant candidates from QU. Only one candidate, *E8-16*, had consistently and substantially higher IR (i.e., WMTM consumed more *Pt x Pa* wildtype leaves than mutant leaves). *E8-16* was also validated as an IR mutant by having a negative effect on WMTM larval weight gain and development in no-choice bioassays.

A new screen of MTU mutants was conducted to identify additional mutants with altered WMTM feeding preference in leaves before and after wounding. We screened 770 mutants and identified approximately 5% to be potentially altered in IR compared to *Pt x Pa* trees. Between the QU validation and the MTU screen we have identified several mutants that show an altered WMTM feeding preference that can be further validated

with additional bioassays and eventual molecular work designed to map T-DNA insertions and identify genes responsible for the phenotype. Results for all bioassay experiments can be found in chapter 3. Ultimately we decided to continue with objectives #2 and #3 using the IR mutant *E8-16*.

Objective #2: Map the T-DNA Insertion and Identify the Activated Gene in the AT Mutant *E8-16*.

The *E8-16* mutant was created in the Regan Lab (Harrison et al. 2007) by inserting the pSKI074 AT vector (Weigel et al. 2000) into a *Populus tremula x P. alba* clone 717-1B4 (*Pt x Pa*) background using *Agrobacterium*-mediated transformation (Han et al. 2000; Tzfira et al. 1997). The vector pSKI074 contains four copies of a cauliflower mosaic virus (CAMV) enhancer that can act upon a nearby gene promoter and "activate" expression of an endogenous gene roughly 5-10 fold above wild-type levels (Weigel et al. 2000). Because AT is a random insertional mutagenesis approach it requires a means to identify where the T-DNA(s) have inserted within the *Populus* genome and to evaluate expression of genes near the T-DNA site to identify which gene(s) are impacted by the CAMV enhancers. This validation is necessary to confirm that the observed phenotype is caused by the AT mutation and not by random mutations created during the tissue culture process to generate the AT trees.

To accomplish objective #2, two PCR methods, SiteFinding (Tan et al. 2005), and thermal asymmetric interlaced (TAIL) PCR (Busov et al. 2010; Liu et al. 1995) were used to amplify the region of the *Populus* DNA next to the left border of the T-DNA vector within the *E8-16* genotype. A basic local alignment search (BLAST) of multiple PCR products sequenced from these two methods within the *Populus trichocarpa*

genome (Phytozome, www.phytozome.net) identified the same genomic region within chromosome 10. Most T-DNA insertions in previous AT *Populus* research were found in intergenic regions of the genome and activated a gene within 13 kb of the mutation (Busov et al. 2010). Since the T-DNA location we mapped for *E8-16* was located outside predicted gene models in the *Populus trichocarpa* genome, it suggested that the T-DNA had not disrupted a gene and that nearby genes should be tested to determine which gene(s) were being "activated". To identify the "activated" gene, expression of three genes located near the T-DNA site was measured using quantitative PCR (qPCR) in *E8-16* and *Pt x Pa* wild-type leaves. The gene model POPTR_10s12800 (*10s12800*), located closest to the T-DNA insertion site, showed a nearly seven-fold increase in expression compared to the wild-type trees. *10s12800* appears to be the gene activated by the T-DNA enhancers. Results from the T-DNA mapping and gene identification can be found in chapter 4.

Objective #3: Characterize the Function of *10s12800*, the Putative E3 RING-H2 Ubiquitin Ligase Gene Found to be Over-expressed in the *E8-16* mutant.

With the identification of the activated gene in *E8-16*, the next steps in a forward genetics approach involve characterizing the function of the gene of interest and confirming that the mutation is indeed causing the phenotype of interest. In particular we were interested in answering questions related to: 1) potential protein function of 10s12800, 2) where the gene is expressed, and 3) identifying potential defensive pathways influenced by increased *10s12800* expression in the *E8-16* mutant.

To identify potential function, we conducted a bioinformatics analysis of the amino acid sequence of 10s12800. To determine the class of the protein, we conducted a

BLASTP search of the non-redundant database within the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov). Orthologs were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2; Larkin et al. 2007) and a boxshade plot was created to identify conserved regions within the group of proteins. Transmembrane domains were identified using the ARAMEMNON database (http://aramemnon-botanik.uni-koeln.de, Schwacke et al. 2003) and InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan; Quevillon et al. 2005) and PROSITE (http://prosite.expasy.org; Sigrist et al. 2010) were used to identify functional domains of 10s12800. We found 10s12800 to be a predicted E3 ubiquitin ligase with a conserved, really interesting new gene (RING) H2 domain with two predicted transmembrane domains near the N-terminus.

E3 ligases function as protein regulators within the ubiquitin/proteasome pathway. Ubiquitination is a multistep reaction that involves three enzymes to tag proteins with ubiquitin, a 76 amino acid regulatory protein, for degradation: ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3) (Glickman and Ciechanover 2002). Within *Arabidopsis*, nearly 5% of all genes are predicted to be involved in the ubiquitin-26S proteasome pathway, suggesting that protein ubiquitination is essential in a number of biological processes for plants (Smalle and Vierstra 2004). RING E3 ligases contain a domain of 40-60 amino acids that bind two atoms of zin. This domain may function as a scaffold in protein-protein interactions to bring the E2 with thioester-linked ubiquitin and a specific protein substrate together for ubiquitination (Lorick et al. 1999). RING proteins have been associated with a number of regulatory pathways (Chen and Hellman 2012) as well as environmental interactions

(Schweichheimer et al. 2009). RING E3 ligases are continually being discovered in forward genetic screens (Bueso et al. 2014; Huang et al. 2010). Therefore it comes as no surprise that we have identified an E3 ligase that may be involved in plant defense against an insect herbivory.

Understanding where the gene is expressed within tree tissues can also lead to clues about gene function within the plant. If gene expression is limited to one tissue type, it may suggest a specific role in the development and regulation of those tissues. To understand where the gene is expressed within *Pt x Pa* trees, we used qPCR to measure *10s12800* abundance on a tissue panel consisting of mature leaves, immature leaves, phloem, bark, xylem, petioles, and roots. Constitutive expression from the tissue revealed *10s12800* is ubiquitously expressed in all tissues with greatest expression in mature leaves.

To identify potential defensive pathways influenced by increased *10s12800* expression, we analyzed the leaf transcriptomes of *E8-16* and *Pt x Pa* wildtype trees using an Agilent 4x44K microarray. RNA was isolated and then sent to our collaborator, Dr. Matias Kirst at the University of Florida, for cDNA synthesis and Cy3 and Cy5 labeling before being hybridized to the *Populus* microarray. Microarray analysis using a gene-by-gene mixed ANOVA model in SAS9.2 revealed only 22 out of 43,803 probes with differential expression using the criteria of $p<0.05$ and a false positive $q<0.15$. The genes over-expressed in *E8-16* included *10s12800*, bZIP and MADS-BOX transcription factors, and chitinases. We hypothesize that it is the over-expression of class I chitinases that may be causing the IR phenotype, possibly by disrupting the peritrophic membrane of the insect gut.

One of the most important aspects of forward genetics is creating new transgenic trees to recapitulate the phenotype observed in the initial mutant as a "gold standard" that the gene is indeed responsible for the phenotype. Our collaborators at North Dakota State University, Dr. David Dai and Danqiong Huang, created new transgenics by inserting an additional copy of the *10s12800* gene using a pCAMBIA S1300 vector into the *Pt x Pa* and *P. grandidentata x P. canadensis* (*Pc x Pg*) backgrounds. Thirteen *Pt x Pa* and nine *Pc x Pg* lines were generated, brought back to UND, and screened for *10s12800* expression. We used qPCR to identify one transgenic line, *TL4,* in the *Pt x Pa* background with two-fold over-expression of *10s12800* compared to empty vector controls (Nested ANOVA, $p<0.05$, $n=5$). Choice assays revealed that WMTM preferred to consume vector control trees compared to *TL4* (Nested ANOVA, $p<0.05$, $n=8$ pairs). However, we did not see a statistical difference in insect growth and development in a no-choice assay where larvae were caged on either *TL4* or vector control trees (t-test, $p>0.05$, $n=9$).Additional transgenic lines are being created with the hope of achieving the same fold difference in *10s12800* expression as the original *E8-16* mutant. These new mutants will also be tested using bioassays to confirm the gene's impact on IR. Results for objective #3 can be found in chapter 4. Chapter 5 provides a summary of the data and suggests some possible future directions to further characterize the function of the *10s12800* gene.

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CHAPTER II

LITERATURE REVIEW

Introduction

Trees are important ecological and economical resources. For over 370 million years, trees have dominated the terrestrial landscape providing habitat for numerous species as well as serving an important role in regulating planetary atmospheric conditions through carbon sequestration. As an economic resource, the U.S. forest industry has become a $200 billion industry providing timber, paper, and renewable fuel as well as employing approximately one million workers (www.epa.gov/agriculture/forestry.html). Because of an ever-increasing demand for tree products, there is great interest in determining genes involved in various tree biology traits, including IR, to develop superior trees for plantation forests.

Until the last decade our ability to potentially improve tree stocks for IR using technology was limited due to a lack of functional genomic tools such as sequenced genomes and mutagenized populations for rapid screening to identify defense-related genes and gene regulatory networks. With the sequencing of the *Populus* (*Populus trichocarpa,* Torr & Gray) genome (Tuskan et al. 2006) and the generation of AT mutant populations (Busov et al. 2010; Harrison et al. 2007), we can now identify genes and gene regulatory networks that cause IR. This chapter will serve as a literature review

to: 1) introduce the genus *Populus* and its ecological and economic value, 2) describe why *Populus* can be a good model for studying perennial plant-insect interactions, 3) introduce important lepidopteran defoliators of *Populu*s, and 4) discuss known *Populus* defenses in response to insect defoliators and the impact of scientific and technological advances have had in identifying potential defense related genes

Ecological and Economic Importance of *Populus*

The genus *Populus* (cottonwoods, poplar, aspen) includes 30-40 species that span the entire Northern Hemisphere. Hybridization within the genus frequently occurs where ranges overlap and is likely to have been a major driver in the speciation of *Populus*. This has resulted in some problems in creating taxonomic groupings of *Populus* (Eckenwalder 1996, Hamzeh and Dayanandan 2004). Many *Populus* species serve important ecological roles as "foundational species" in early successional forest ecosystems due to their ability to rapidly colonize disturbed areas in riparian and recently burned habitats. Most *Populus* species have adapted to live in riparian and wetland habitats where seasonal flooding can occur. Riparian-dwelling species, such as *Populus nigra* (Linneaus), are highly adapted to water dynamics and sediment movement allowing them to colonize riverbanks (Storme et al. 2004). Seeds can be quickly dispersed by wind and water and can establish along riverbanks on sites that have experienced natural winter floods. Moist silt and sand, following the recession of flooding, create an ideal environment for the germination of seeds and tree establishment (Vanden Broeck 2003). *Populus* can also produce clones by vegetative propagation from broken twigs and branches that can take root on riverbanks (Braatne et al. 1996) or by root suckering from exposed roots or roots near the soil surface (Barnes 1966). Eliminating natural flooding by damming rivers or diverting

rivers has been shown to negatively impact *Populus* regeneration in riparian ecosystems (Braatne et al. 1996; Vanden Broeck et al 2003).

Figure 1: Eastern cottonwood (*Populus deltoides* Bartram ex Marshall) along the Minnesota River near St. Peter, MN.

Other *Populus* species are better adapted to drier, upland environments. For example, *Populus tremula* (Linnaeus), commonly referred to as the Eurasian aspen or common aspen, grows best in moist, well-aerated soil. However, it can grow in a variety of soil types, tolerate seasonal flooding or up to two months of less than 40 mm of rainfall, and can grow in elevations ranging from sea level to 1600 meters (von Wuehlisch 2009). *Populus tremuloides* (Michaux), a popular species used in research, is the most widespread tree species in North America primarily found in Canada, around the

Great Lakes, and in the arid mountain west region of the U.S. (Perala 1990). This species can survive in a variety of soils (Perala 1990) and regularly colonize unstable environments such as volcanic cones, rock outcrops, glacial outwash, and landslides. *Populus tremuloides* (Michaux) communities in the mountain west region are found in mid to high elevations with annual temperature extremes ranging from -57° to 41°C (Perala 1990). This species can be found scattered within montane and subalpine conifer forests but can also be found in extensive pure stands within the southern Rocky and Utah ranges (Shinneman et al. 2013). Fire has been shown to have an important role in regeneration of this species (Shinneman et al. 2013). Vegetative root suckering, along with some regeneration from wind-borne seeds, allow for the rapid colonization of disturbed areas following forest fires.

Besides their ecological importance, *Populus* also have substantial economic value. Characteristics of the genus, such as fast growth, easy propagation, and appearance have made it a desirable wood source for landscape and agricultural purposes for thousands of years. In fact, the word *Populus* comes from the term *arbor populi*, or "the people's tree", for its widespread use during the Roman Empire (Gordon 2001). Recent archeological evidence suggests *Populus* was used for heating and house construction as far back 10,000 years ago along the Euphrates River in the Middle East(Settler 2009). Other early cultures, such as the Objibwe in North America (8000 B.C.), the Neo-Sumerian Empire in Mesopotamia (2100 B.C.), Chinese (700 B.C.), and the Hohokam natives of Mexico (800 A.D.), all used *Populus* for purposes, including cooking, heating, construction/shelter, medicine, tools, soil stabilization, and windbreaks (Gordon 2001; Stettler 2009; Hageneder 2005; Logan 2002).

Exploitation of natural forests during WWII, as well as demand for lumber products during post-war industrialization, drove the development of industrial plantation forests in Europe (FAO 1980, Castro and Zanuttini 2008). In the U.S., plantation forestry expanded in the late 20th century due to depletion of natural stands of *Populus* as a result of agricultural conversion and damming and diverting natural river flows (Sternitzke 1976). This, combined with the increasing human population in the later half of the 20th century, resulted in the need for plantation forestry and sustainable forest industry practices to meet demand for wood products (Sternitzke 1976). By the end of the 20th century concerns over sustainability of forests had become a major global concern and was a significant topic at the 1992 United Nations Conference on Environment and Development (Sedjo et al. 1998).

Today many countries have extensive plantations of *Populus,* with the largest found in China (7.6 million ha), France (236,000 ha), Iran (150,000 ha), and Turkey (125,000 ha) (FAO 2008; FAO 2012; Stanturf and van Oosten 2014). *Populus* make up the largest fraction of managed hardwood forest acreage in the U.S. and Canada (Coyle et al. 2005). Approximately 45,000 ha of *Populus* species are grown in the U.S. primarily for the production of bioenergy, pulpwood, lumber, and paper products (Stanturf and van Oosten 2014). *Populus* are also seen as a potential source for cellulosic ethanol, with significant work being done to reduce or change the lignin content of these species to make the process more economical (Meyermans et al, 2000; Anterola and Lewis 2002; Sasaki et al. 2004). *Populus* are also cultivated for a number of environmental applications. *Populus* are commonly used as windbreaks and shelterbelts designed to alter wind flow around farmsteads and homes. They are also useful for preventing soil erosion

and as riparian buffers. Because *Populus* species grow rapidly and have root systems capable of taking up a lot of water and nutrients (Isebrands and Karnosky 2001; Licht and Isebrands 2005) they have also been used for phytoremediation, the cleaning up of contaminated soil and water by removing and degrading toxic chemicals using plants, as well as carbon sequestration (Coyle et al. 2005).

One of the challenges for plantation forests, as well as natural forests, is that they are prone to substantial insect herbivory. Dense populations of *Populus* with limited genetic diversity can create spatially uniform, low biodiversity environments vulnerable to insect outbreaks (Neuvonen and Niemela 1983; Niemela and Neuvonen 1983; Mattson et al. 1991; Haack and Mattson 1993). Unfortunately, some tools used for pest control in annual agricultural crops are are not suitable for application in forestry. For many agricultural crops, pesticide applications combined with annual crop rotations are effective in reducing insect herbivory. However these are not viable solutions in wide-scale forests where trees are grown over a number of years (Phillipe and Bohlman 2007). Despite the potential use of genetic engineering of pest resistance in trees, so far the deployment of genetically modified trees has been prevented in many jurisdictions (Lida et al. 2004, Phillipe and Bohlman 2007) due to the possibility that transgenic populations will breed with wild trees and concerns over unknown impacts on the environment. Domestication, defined as the process of exploiting and manipulating genetic variation at multiple levels to breed commercial cultivars for wood-based commodities, energy feedstock, and environmental services (Libby 1973; Dickmann et al. 1994; Bradshaw and Strauss 2001), of *Populus* has been on-going for 100 years (Stanton et al. 2014). As a fast growing wood source that can be hybridized with relative ease, *Populus* has become a

useful organism for selecting trees for improved growth, adaptability and increased resistance to biotic pests for forest plantations (Broderick 2010, Coyle et al. 2005). Traditional domestication of *Populus* involved interspecific hybridization by combining genomes of distinct species, selecting clones with ideal traits, and using vegetative propagation to maintain genetic lines for planting and further testing or for additional genetic crossing (Stanton et al. 2014). Unfortunately, without the aid of modern molecular techniques, this approach requires extensive testing and breeding in an organism that typically requires 7-10 years before each generation develops flowers. As a result, traditional breeding strategies are slow and there is limited information about what genes are contributing to the phenotype.

While extensive deployment of transgenic trees may not be an option in the near future, molecular approaches have been developed that allow scientists to more quickly identify genes and allelic combinations for desirable traits. Genome wide association studies using DNA-based markers such as restriction fragment length polymorphisms (RFLP), amplified fragment length polymorphism (AFLP), randomly amplified polymorphic DNA (RAPD), microsatellites, and functional markers such as single nucleotide polymorphisms (SNPs), along with genetic transformation studies have greatly contributed to our understanding of genes associated with *Populus* traits (Bradshaw et al. 1994; Schroeder et al. 2012; Lojewski et al. 2009; Cervera et al. 2005). These tools have allowed researchers to identify genes to target for breeding programs to develop superior trees. The following sections will discuss why *Populus* is a good model for studying plant-defoliator interactions, common lepidopteran pests to *Populus*, and what prior

research has been done to identify genes involved in IR so that *Populus* breeders can incorporate this information into breeding programs to produce superior IR trees.

Populus as a Model Organism for Tree Biology

Populus has become a model system for studying aspects of deciduous tree physiology including wood formation, environmental adaptation, and responses to biotic stresses including pathogens and insect herbivores (Mauriat et al. 2015; Philippe and Bohlman 2007; Jansson and Douglas, 2007; Cronk, 2005; Brunner et al. 2004). Characteristics that make this an ideal tree species to study include: fast growth, short reproductive cycle for trees (7-10 years), relatively easy to genetically modify (Chupeau et al. 1994; Confalonieri et al. 1995; Qiao et al. 1997; Ma et al. 2004; Takata and Eriksson 2012), enormous size for spatial studies, and it was the first tree species to have a sequenced and annotated genome (*Populus trichocarpa*; Tuskan et al. 2006). The genome size is relatively small in terms of trees, with the haploid genome size approximately 485 million base pairs (Tuskan et al. 2006), only four times larger than *Arabidopsis*. The genome also contains approximately 45,000 coding genes, most with some similarity to the *Arabidopsis* genome (Tuskan et al. 2006). The smaller genome makes *Populus* an attractive target for map-based cloning of genes. Numerous tools have been created to allow for large genome wide expression studies of *Populus* including development of microarray platforms (Ralph et al. 2006; Harding et al. 2005; Brosche et al. 2005; Andersson et al. 2004) large collections of expressed sequence tags and full-length cDNAs (Ralph et al. 2008; Ralph et al. 2006; Sterky et al. 2004; Christopher et al. 2004; Ranjan et al. 2004), RNA-seq data (Ariani et al. 2015; Liu et al. 2014; Zhang et al. 2014) and proteomic and metabolite studies (Hamanishi et al. 2015; Nilsson et al. 2010;

Kieffer et al. 2009). Research prior to the creation of these molecular tools focused on the study of tree-insect interactions at the physiological and ecological levels. These newer molecular tools now allow us to explore tree-insect interactions and the diverse anatomical structures and chemical plant defenses used to combat insect herbivory at the molecular and cellular/biochemical levels.

Lepidopteran Defoliators of *Populus*

Long lifespan (up to 400 years), large size, sessile lifestyle, late sexual reproduction, and their ecological dominance expose *Populus* to a wide variety of more rapidly evolving insect herbivores. Approximately 300 species of insects and mites in North America consume *Populus* (Mattson et al. 2001). Of the hundreds of insects that feed on *Populus*, less than 10% create enough damage to be considered serious pests. Insects found on *Populus* species, grouped by feeding lifestyle, include gall formers, sucking/phloem feeders, wood borers, root feeders, and leaf feeders (which include defoliators and leaf miners). Table 1 shows some insect species for each lifestyle group. Gall formers, such as *Saperda*, *Dasineura*, and *Prodiplosis* species, produce galls when feeding on shoots, petioles, or leaf lamina. The galls provide young instars food as well as protection from predators (Charles et al. 2014). Sucking aphid species, such as *Phloemyzus passerinii* (Signoret) and *Tuberolachnus salignus* (Gmelin) contain mouthparts called stylets designed to puncture bark and suck phloem. Wood boring and leaf feeding insects are often considered pest species. Wood-boring insects can cause substantial damage to the trunk making the trees unsuitable for certain wood products and more vulnerable for collapse under heavy winds (Charles et al. 2014). Defoliating insects comprise the largest group of insect pests for *Populus* and primarily come from the

Table 1: List of some insect species that feed on *Populus* trees. Table shows some representatives from Hempiptera, Coleoptera, Lepidoptera, Hymenoptera, and Diptera along with their common names and feeding style.

Species	Common Name	Feeding Style
Hemiptera		
Chaitophorus leucomelas (Koch)	None (aphid species)	Shoot/phloem sucking
Parthenolecanium corni (Bouché)	European fruit lecanium	Shoot/phloem sucking
Phloemyzus passerinii (Signoret)	Woolly poplar aphid	Shoot/phloem sucking
Tuberolachnus salignus (Gmelin)	Giant willow aphid	Shoot/phloem sucking
Coleoptera		
Agrilus liragus (Barter and Brown)	Bronze poplar borer	Wood Borer
Anaplophora glabripennis (Motschulsky)	Asian longhorned beetle	Wood borer
Chrysomela populi (Linnaeus)	Poplar leaf beetle	Leaf feeder
Cryptorhychus lapathi (LeConte)	Poplar and willow borer	Wood borer
Gypsonoma haimbachiana (Kearfott)	Cottonwood twig borer	Wood borer
Oberia schaumii (LeConte)	Poplar branch borer	Wood borer
Polydrusus impressifrons (Gyllenhal)	Pale green weevil	Root feeder, Leaf feeder
Polydrusus sericeus (Schaller)	Green immigrant leaf weevil	Root feeder, Leaf feeder
Saperda calcarata (Say)	Poplar borer beetle	Gall former
Saperda inornata (Say)	Poplar-gall saperda	Gall former
Lepidoptera		
Choristoneuria conflictana (Walker)	Large aspen tortrix	Leaf feeder
Clostera inclusa (Hübner)	Poplar tent maker	Leaf feeder
Lymantria dispar (Linnaeus)	Gypsy moth	Leaf feeder
Malacosoma disstria (Hübner)	Forest tent caterpillar	Leaf feeder
Orgyia leucostigma (J.E. Smith)	White-marked tussock moth	Leaf feeder
Phyllocnistis populiella (Chambers)	Common aspen leaf miner	Leaf feeder
Hymenoptera		
Janus abbreviates (Say)	Willow shoot sawfly	Leaf feeder
Trichiocampus viminalis (Fallen)	Poplar sawfly	Leaf feeder
Tremex fuscicornis (Fabricius)	Tremex wasp	Wood borer
Diptera		
Dasineura populeti (Rübsaamen)	Gall midge	Gall former
Prodiplosis morrisi (Gagne)	Poplar gall midge	Gall former
Phytobia cambii (Hendel)	Poplar cambium mining fly	Wood borer

Coleoptera and Lepidoptera taxanomic groups (Harrell et al. 1981, Mattson et al. 2001). Because this dissertation is concerned with identifying *Populus* genes involved in deterring lepidopteran larval insect defoliation, this literature review will cover lepidopteran herbivory and some of the more common lepidopteran defoliating pest species in more detail.

Some of the more important lepidopteran defoliators of *Populus* in the U.S. and Canada (figure 2) include the white-marked tussock moth (WMTM, *Orgyia leucostigma*

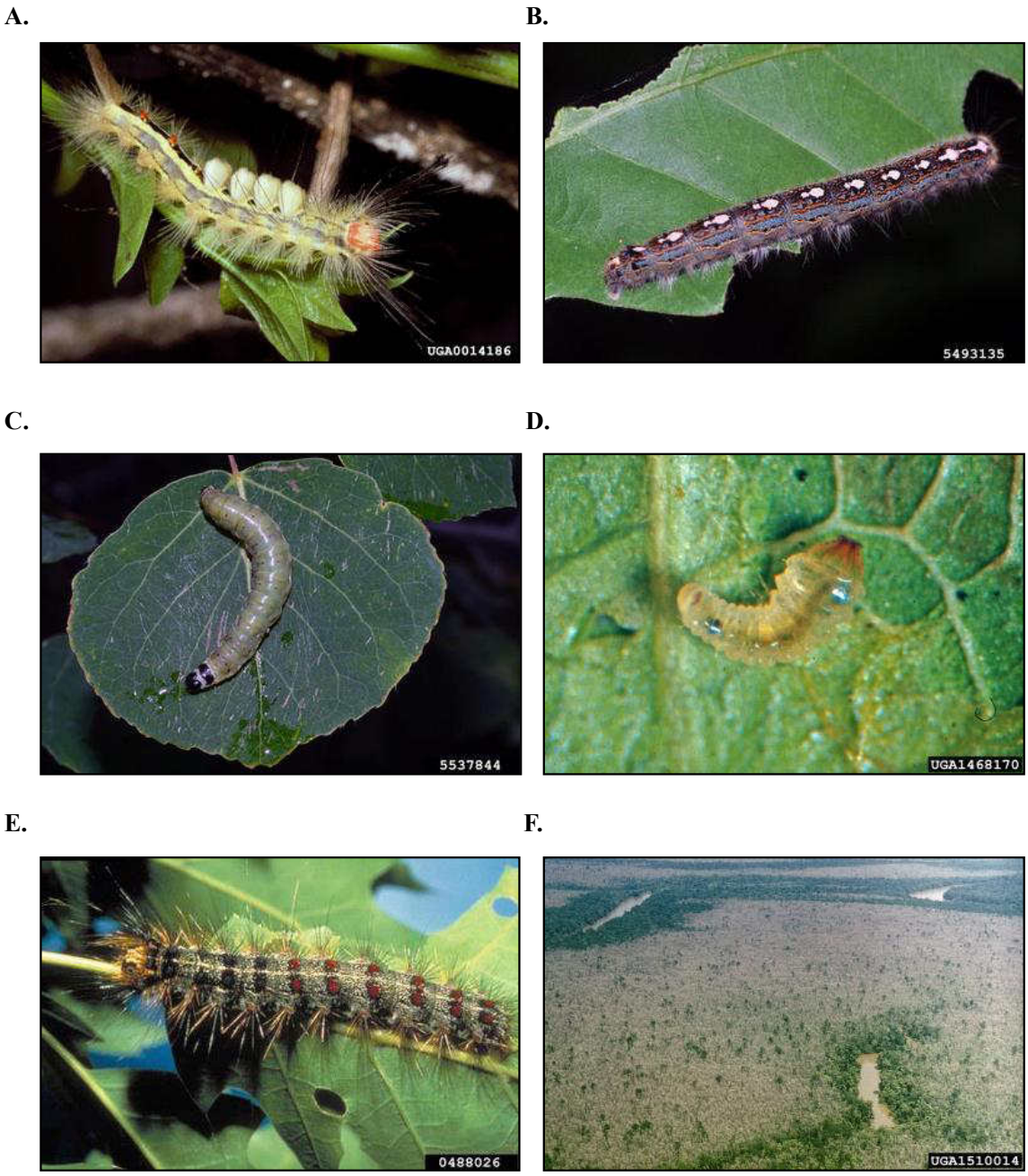


Figure 2: Common lepidopteran defoliators of *Populus.* A) White-marked tussock moth, image by Gerald J. Lenhard, Louisiana State University, Bugwood.org. B) Forest tent caterpillar, image by William M. Ciesla, Forest Health Management International, Bugwood.org. C) Large aspen tortrix, William M. Ciesla, Forest Health Management International, Bugwood.org. D) Common aspen leaf miner, image by USDA Forest Service-Ogden, USDA Forest Service, Bugwood.org. E) Gypsy moth, image by John H. Ghent, USDA Forest Service, Bugwood.org. F) Extensive defoliation by forest tent caterpillars, image by USDA Forest Service-Region8-Southern, USDA Forest Service, Bugwood.org.

J.E. Smith), forest tent caterpillar (FTC, *Malacosoma disstria* Hübner), large aspen tortrix (*Choristoneura conflictana* Walker), common aspen leaf miner (*Phyllocnistis populiella* Chambers) and gypsy moth (*Lymantria dispar* Linnaeus). Lepidopteran defoliators are generally not considered a great threat to tree survival. However, the extent and duration of defoliation can influence *Populus* growth and mortality. Light defoliation has little effect on *Populus* mortality or tree growth (Ives and Wong 1988). Under short periods of extreme defoliation trees can tolerate a significant loss of leaves (Robison and Raffa 1994; Reichenbacker et al. 1996). For example, Robison and Raffa (1994) they tested clones of fifteen hybrid *Populus* against FTC using laboratory and greenhouse trials to examine relationships between tree productivity, defoliation tolerance, and insect resistance. Despite 75% defoliation several of the hyrids displayed substantial defoliation tolerance and tree productivity.

Extensive outbreaks of native insect species, or the accidental release of non-native species, can significantly reduce biomass production and wood quality in *Populus* (Reichenbacker et al. 1996). Wagner and Doak (2013) used insecticide on quaking aspen at two sites in Alaska over a period of seven years during an aspen leaf miner multi-year outbreak period and compared tree growth to non-treated aspen. By the seventh year ramets with ambient levels of leaf mining possessed half the leaves and fewer than half the shoots than ramets that were treated with the insecticide. Control trees also displayed smaller leaves, reduction in height, and reduction in girth. They also observed greater dieback to basal sprouts in control trees than those protected by the insecticide suggestingthat insect defoliation can greatly impact health and productivity of *Populus*.

Lepidopteran defoliators pose a greater risk to *Populus* species during periodic population outbreaks, when substantial defoliation over consecutive growing seasons weakens the tree resulting in the increased incidence of fungal disease and infestation by opportunistic insects (Churchill et al. 1964; Klepzig et al. 1997; Hogg et al. 2002), especially when coupled with abiotic environmental stresses. In Hogg et al. (2002) they found that die-off in trembling aspen in western Canada in the 1990's was likely due to the result of substantial defoliation events from FTC coupled with drought in 1960's and 1980's that led to reduced growth and susceptibility to wood boring insects such as *Saperda calcarata* (Say), and *Agrilus liragus* (Barter and Brown) and fungal pathogens (*Armillaria spp*, *Phellinus tremulae*, *Venturia macularis*, *Hypoxylon mammatum*, *Peniophora polygonia*). Weakening of trees for secondary opportunistic insects and pathogens can cause widespread economic loss in *Populus* plantations (Harrell et al. 1981; Coyle et al. 2002; Hogg et al. 2002). To minimize substantial lepidopteran defoliation of natural and plantation forests we need to understand what gene regulatory networks are involved in plant defense to minimize substantial lepidopteran defoliation. The next few sections will describe the common lepidopteran defoliators of *Populus* in more detail.

White-marked Tussock Moth

WMTM is the insect species we use to study plant-insect interactions, as it is considered a top three defoliator of *Populus* species in the U.S. and Canada. The WMTM has wingless female moths that lay 100-300 eggs in a single cocoon from which offspring emerge (Wagner 2005). The light brown larvae emerge in the spring and feed on the undersides of young foliage of over 140 different plants including *Populus* species

(Wagner 2005). Because this species can consume leaf material from many different plants sources they are considered generalist herbivores. Young larvae can be dispersed to neighboring vegetation by "ballooning" (Wagner 2005). "Ballooning" refers to the wind breaking threads of newly hatched larvae hanging from trees and carrying larvae to other nearby plants. Later instars develop white tufts and gain yellow, red, and black body coloration and become voracious leaf consumers (Rose and Lindquist 1982). Once mature the larvae pupate and then emerge as winged males or flightless females a few weeks later (Martineau 1984; Johnson and Lyon 1991). WMTM exist in a range from the southeastern U.S. and along the U.S. coast into Canada and as far west as Texas, North Dakota, and Manitoba (Wagner 2005). The insect's high fecundity and dispersal behavior can result in significant population outbreaks as well as the establishment of new colonies (Harrison 1997; Maron et al. 2001; van Frankenhuyzen et al. 2002; Yoo 2006). WMTM can infest crops near wooded areas during outbreaks (Howard 1896; Dustan 1923; Isaacs and Van Timmeran 2009). WMTM also contain hairs that can cause rashes for humans, especially during population outbreaks. Crashes in population are typically the result of diseases such as nuclear polyhedrosis (Cunningham 1972) and cytoplasmic polyhedrosis viruses (Hayashi and Bird 1968) and predation from natural enemies such as birds and parasitoids (Howard 1897; Embree et al. 1984; Cunningham and Kaupp 1995; van Frankenhuyzen et al. 2002).

Forest Tent Caterpillars

The FTC is another major native generalist defoliating pest of *Populus* species in the USA and Canada. FTC have a single generation each year that hatch early in the spring with the flushing of foliage. If hatching occurs before foliage is available, larvae

become vulnerable to freezing and starvation (Blais et al. 1955; Raske 1975). Newly hatched larvae form colonies that stay together through the first four instar stages by laying silken trails for larvae to follow others and form silken mats for insects to congregate for resting and molting (Fitzgerald 1995). After two months of feeding on leaves fifth instar larvae have pale blue lines along the sides of their brown bodies with white spots along their back. Larvae pupate five to six weeks following hatching and emerge ten days later as short-lived adults. Adults lay between 100-350 eggs that develop into larvae and overwinter in the eggs until spring (Fitzgerald 1995). During population outbreaks that occur every ten years, FTCs commonly defoliate trees occurring over millions of hectares, with a density as high as 20,000 caterpillars per tree (Stairs 1972; Fitzgerald 1995). As a result FTC larvae can grow to over 1000 times their mass at hatching and consume more than 15,000 times their initial body weight in leaf tissue (Fitzgerald 1995). Light defoliation has little effect on tree growth, however, more substantial feeding over multiple years can result in depressed radial growth at stump and mid crown height (Hildahl and Reeks 1960) and can make trees more susceptible to impacts from other forms of stress (Churchill et al. 1964).

Large Aspen Tortrix

The large aspen tortrix is a native insect species that feeds primarily on *Populus tremuloides* forests north of the range of FTC (Mattson et al. 2001). Newly hatched larvae emerge in mid to late July appearing yellow-green with a light brown head and anal plate (Prentice 1955). First instar larvae feed on the leaf epidermis of expanded leaves before they spin hibernacula for the winter (Mattson 2001). Second instar emerge in the spring and mine *Populus* buds through the third instar. Late instar larvae change

their feeding strategy by webbing expanded leaves together for protection during feeding (Charles et al. 2014). By the fifth instar they appear dark green with a black head capsule, setal bases, and anal plate. Fifth instar larvae undergo pupation for one to two weeks in late June to July and then have approximately five to ten days to mate and lay eggs. During severe outbreaks the species has defoliated trees over an area as large as 1.3 million hectares (Beckwith 1968). Outbreaks normally collapse within two or three years (Cerezke and Volney 1995) due to natural predators and parasitoids.

Common Aspen Leaf Miners

Common aspen leaf miner is found in the northern part of North America including Canada and Alaska. Adults emerge in the spring after spending the winter under the bark of hardwood and conifer trees to feed and mate (Kruse et al. 2007). Adults lay a single egg on newly emerged *Populus* leaves and protect them by folding the leaf over over egg. Small white larvae emerge from the eggs, bore into the leaf, and then feed on the mesophyll layer located between the upper and lower epidermal layers (Kruse et al. 2007). Over a couple months feeding produces serpentine mining pattern through the leaf. Larvae undergo four instar stages and pupate within the mines before emerging as adults in late August and September (Kruse et al. 2007). In 2006 a significant outbreak occurred in Alaska and over a two-year span 305,200 ha were affected (U.S. Forest Service, 2006, 2007). Leaf mining can causes damage to *Populus* by reducing photosynthesis and growth and causing early leaf abscission (Wagner et al. 2008).

Gypsy Moth

Gypsy moth, a non-native lepidopteran species accidentally introduced to the USA in the late 19th century, has become one of North America's most devastating forest

pests. They can successfully consume over two hundred different plant species including *Populus* (McFadden and McManus 1991). Plant host availability, limited natural enemies, and the fact that it can probably survive anywhere in the USA (Giese and Schneider 1979), make the gypsy moth a threat to *Populus* plantation forests in North America. Gypsy moth adult females can deposit up to 1,000 eggs in an egg mass. Newly hatched larvae disperse by "ballooning" and begin feeding when they encounter an acceptable food source. Insects undergo four larval instar stages before transforming into pupae sometime in late June or July. Male moths typically emerge first and begin to fly to find a mate. Adult female moths have well-developed wings but are unable to fly. Females use the sex pheromone disparlure, 2-methyl-7R, 8S-epoxy-octadane, to attract adult males (Bierl et al. 1970). After mating females uslayy lay eggs in the same location where she pupated. Since its introduction in 1979 it has gradually expanded its range (Liebhold et al. 1992) and can now be found as far west as Minnesota. Millions of acres of trees each year have been defoliated since its introduction and the continued expansion of the species threatens vulnerable hardwood populations despite several large-scale barrier programs (McFadden and McManus 1991).

Populus Defenses Against Insect Herbivory

Terminology and Overview of *Populus* Defenses to Lepidopterans

Insect herbivory is a major stress on plants. Due to their sedentary lifestyle trees cannot escape their environment. To deter insects from feeding, trees have evolved a suite of secondary metabolites, biochemical defenses, physical defenses, and indirect defenses to deter or reduce defoliation and make the host plant more insect resistant. Defense, as defined by Karban and Baldwin (1997) and Strauss and Agrawal (1999),

refers to plant traits that provide a fitness benefit to the plant in the presence of insect herbivores. A defense therefore may or may not harm the insect herbivore. IR, however, refers to plant traits that reduce performance or preference of herbivores. Plants respond to insect herbivory through chemical and physical features designed to limit the feeding insects directly (direct defenses) or through tritrophic interactions initiated by the release of volatile bouquets to attract predators of the attacking insect (indirect defenses, Howe and Jander 2008).

Defenses against insects are costly (Baldwin 1998; Mauricio 1998; Koricheva 2002; Strauss et al. 2002) and require trees to balance resource allocation between the growth and development of the tree and defending it from insect herbivores. Therefore, plants, such as *Populus*, have evolved a combination of constitutive and inducible defense strategies to limit insect feeding. Constitutive defenses involve anatomical features and chemical compounds that are present regardless of whether or not insect herbivores are on the tree (Howe and Jander 2008). Constitutive defenses serve as a primary level of defense against insect herbivores. Inducible defenses are only expressed in the plant in response to the presence of a particular stress (Howe and Jander 2008) and are under tight regulatory control (Philippe & Bohlmann 2007; Kessler and Baldwin 2002) as these responses divert resources away from primary processes like photosynthesis (Mattson and Palmer 1988). Induced defenses can be activated locally, or systemically following wounding and insect feeding (figure 3, Parson et al. 1989; Arimura et al. 2004; Lawrence et al. 2006; Major and Constabel 2006; Babst et al. 2009; Philippe et al. 2010). The signals that activate local and systemic induced genes in *Populus* are poorly understood. However, there is evidence that they may come from

jasmonates by way of the octadecanoid pathway (Havill and Raffa 1999; Constabel et al. 2000; Haruta et al. 2001, Arimura et al. 2004; Philippe et al. 2010). Studies based on *Populus* and other plant species suggest other signals could include sugar sensing (Ehness et al. 1997, Rolland et al. 2002), electrical (Lautner et al. 2005), and volatiles (Frost et al. 2007).

Insect herbivory creates a need for resources to support the production of induced defenses. However, loss of leaf material at the herbivory site means less photosynthesis and less carbon available for assimilation to support plant defenses (Schultz et al. 2013). Therefore, resources need to be reallocated from other existing resource pools to create plant defenses. Demand can be met by increased sink strength, a relative ability to draw in and unload photosynthate, at the site of herbivory (Schultz et al. 2013). Sinks are defined as tissues to which photosynthate is drawn. Sources are mature leaves that create carbohydrates through photosynthesis. Mechanical damage, insect feeding, and jasmonic acid have been shown to increase the sink strength to produce phenolic defense compounds in developing leaves for *Populus* (Arnold and Schultz 2002; Arnold et al. 2004; Babst et al. 2005, Philippe et al. 2010). Leaves neighboring a leaf that has undergone herbivory can also be considered sinks as they can experience increased carbon import for the production of phenylpropanoids (Arnold et al.2004). Young leaves in *Populus* are considered sink materials as they do not undergo photosynthesis and therefore do not have the resources available for their own development, much less for defenses (Arnold and Schultz 2002; Arnold et al. 2004). As leaves mature and become source leaves they lose their ability to increase sink strength (Arnold et al. 2004). The leaf plastochron index (LPI) developed by Larson and Isebrands (1971) has been used to

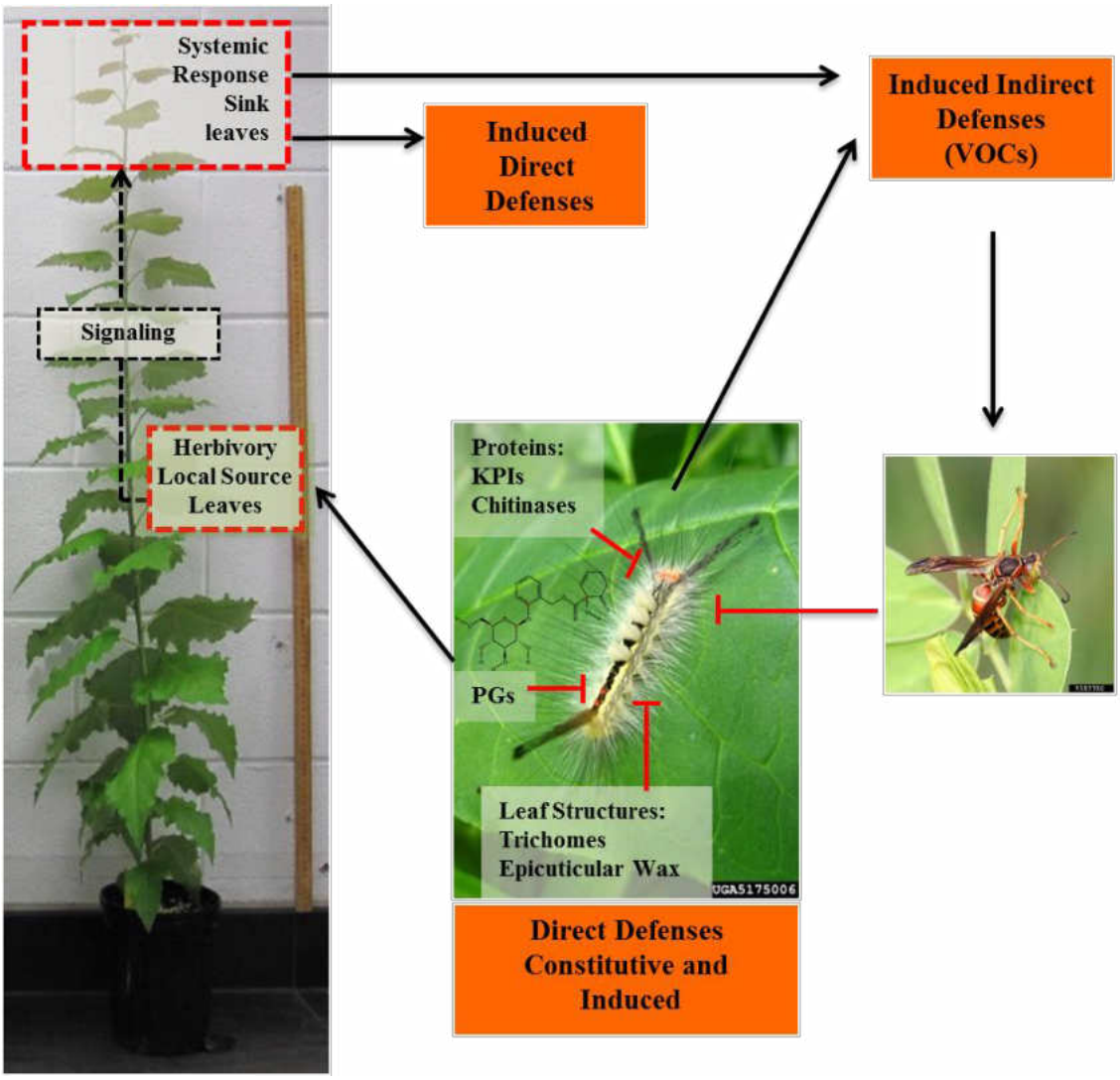


Figure 3: Schematic diagram of *Populus* defenses against insect herbivores. Insect herbivores encounter direct and indirect defenses designed to limit insect feeding. Direct defenses can be constitutive (present regardless of insect presence) or induced upon insect feeding. Some constitutive physical defenses, such as trichomes and epicuticular wax, as well as chemical defenses, such as phenolic glycosides (PGs), serve as a first line of defense against herbivory. Other compounds, such as condensed tannins, kunitz protease inhibitors (KPIs), and chitinases are induced upon insect feeding. Insect presence and feeding can also cause the induction of indirect defenses. The bouquet of volatiles released by injured tissue serves to attract natural predators of the insect herbivore, such as parasitic wasps, to come to the tree. Feeding by insect herbivores not only induces local plant defenses in the wounded leaf, it can also activate systemic sink leaves through signals that either travel through plant through phloem or by volatiles. Image of WMTM comes from Kevin D. Arvin, Bugwood.org. Image of the paper wasp *Polistes fuscatus* comes from Johnny N. Dell, Bugwood.org.

standardize developmental stage of leaves in *Populus* (Philippe and Bohlman 2007). The first leaf that is 2 cm in length is designated as LPI 0 and the transition from sink to source leaves in *Populus* occurs between LPI five and six (Larson and Dickson RE 1973). Another factor that can influence activation of systemic defenses is phloem connectivity between leaves. In a study by Davis et al. (1991) mechanical wounding of LPI 9 resulted in *win3* transcript detection in unwounded portions of the wounded leaf as well as in LPI 4. This study demonstrated that every fifth leaf is connected by phloem and that local wounding could activate defenses in leaves that were connected systemically. The next few sections will discuss specific examples of *Populus* defenses. For additional information on general *Populus* defenses used in response to insect herbivory see Philippe & Bohlmann (2007), Ralph (2009), and Lindroth and St. Clair (2013).

Populus Constitutive Defenses

Physical. Constitutive defenses that serve as physical barriers to directly impede lepidopteran feeding on *Populus* leaves include trichomes (Rudgers et al. 2004) and epicuticular waxes (Eigenbrode and Espelie 1995). Trichomes are enlarged, modified epidermal cells that extend from epidermal layers either as hair-like projections to serve as a barrier to insect feeding, or grow perpendicular to the leaf surface as glandular trichomes that accumulate toxins (Wagner 1991; Mauricio 1998; Simmons and Gurr 2005). As their density increases, trichomes can decrease transpiration rates (Choinski and Wise 1999; Pérez-Estrada et al. 2000) and increase defenses against herbivores and parasites (Neal et al. 1989; Bodnaryk 1996). In a forward genetic screen of AT *Populus* Harrison et al. (2007) identified a mutant, *fuzzy*, with increased trichome density. Plett et

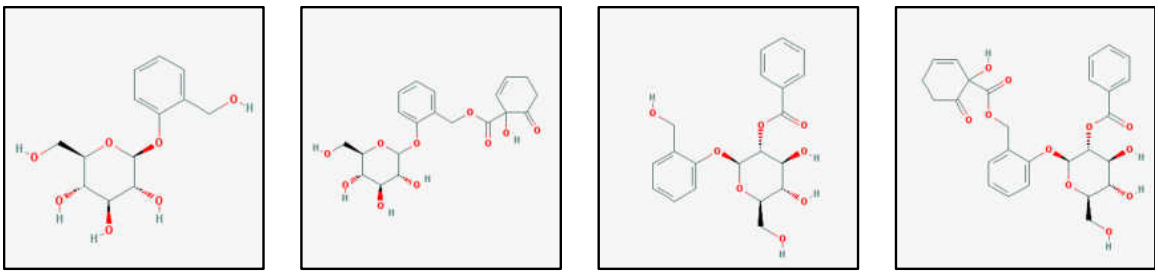
al. (2010) identified the over-expressed gene in the *fuzzy* mutant as a MYB transcription factor and confirmed that greater trichome density in the mutant negatively impacted WMTM larval feeding and development in choice and no-choice bioassays.

Leaf surfaces are covered by a cuticle, consisting of a cutin matrix along with cuticular waxes that form a barrier between the plant and its environment (Jenks and Ashworth 1999; Martin and Juniper 1970). Cuticular waxes are complex mixtures of primary alcohols, fatty acids, and alkyl esters combined with odd-numbered chain length hydrocarbons, secondary alcohols, and ketones (Walton 1990). Waxes inside the cutin matrix are called intracuticular whereas waxes outside the cutin matrix and exposed on the leaf surface are called epicuticular waxes (Jetter et al. 2000). Epicuticular waxes have diverse chemical compositions, and can change in relative abundance depending on plant age and development, and the environment (Jenks and Ashworth 1999). Epicuticular waxes are important for reducing surface transpiration in plants (Jordan et al. 1984; Cameron et al. 2002). Epicuticular waxes have also been shown to have a role in plant defense to insects. For insect herbivores to consume leaf material they must come in contact with the leaf surface and either consume or penetrate through the waxy layer (Southwood 1986; Eigenbrode 1996). The cuticle has been shown to impact insect behavior in a number of studies (Varela and Bernays 1988; Renwick et al. 1992; Griffiths et al. 2000; Eigenbrode and Jetter 2002). Laboratory experiments by Alfaro-Tapia et al. (2007) demonstrated that *Chaitophorus leucomelas* (Koch) devoted less time to probing behavior in resistant hybrids of *Populus* compared to susceptible hybrids. However, when leaves were de-waxed they did not observe a difference in probing behavior time. As a

physical barrier it appears to be effective in negatively impacting *Chaitophorus leucomelas* (Koch) feeding behavior.

Chemical. In addition to physical characteristics, *Populus* also produce constitutive chemical defenses. One of the major classes of secondary metabolites found within *Populus* species are phenols. These compounds are regulated by the shikimate/phenylpropanoid pathways (Philippe and Bohlmann 2007), which produce thousands of chemicals including simple phenols, flavanoids, stilbenes, coumarins, lignans and the salicin-based phenolic glycosides (PGs), hydroxycinnate derivitives (HCDs) and condensed tannins (CTs) (Tsai et al. 2006, Philippe and Bohlmann 2007). Their roles in defense, as well as genes involved in the phenylpropanoid pathway, are covered in reviews by Tsai et al. (2006) and Chen et al. (2009). Only a few of these chemical groups have been studied for their role in plant defense against insects. The most studied example of chemical constitutive defenses in *Populus* are the suite of salicylate-glycosides known as PGs. Biosynthesis of salicylate-derived PGs in *Populus* are poorly understood (Chen et al. 2009). However, it appears this group of compounds is derived from precursors of salicylic acid biosynthesis (Pierpoint 1994; Lee et al. 1995; Dempsey et al. 1999). Salicylic acid can be synthesized from chorismate-phenylalanine via cinnamate and benzoate although it has been shown in *Arabidopsis* that salicylic acid can be synthesized in chloroplasts from isochorismate (Wildermuth et al. 2001; Metraux 2002). Twenty salicylate-derived PGS have been found within the genus *Populus*, however, the best-studied PGs in terms of IR in *Populus* are salicin, salicortin, tremuloidin, and tremulacin (figure 4, Tsai et al. 2006). Phenolic glycoside tissue concentration, like many secondary chemical defenses in *Populus*, are shaped by genetic,

developmental, and environmental factors (Lindroth and Clair 2013). PGs are found



Salicin **Salicortin** **Tremuloidin** **Tremulacin**

Figure 4: *Populus tremuloides* (Michaux) phenolic glycosides. Salicin, salicortin, tremuloidin, and tremulacin are well-studied PGs. Insecticidal activity increases from left (salicin) to the right (tremulacin). Structures come from the National Center for Biotechnology Information (NCBI) PubChem database.

within bark and leaf tissues of *Populus*. Within leaves, salicin and tremuloidin occur in low concentrations, roughly 1%. However, salicortin and tremulacin can be as high as 15% (Hemming and Lindroth 1995; Lindroth and Hwang 1996; Osier et al. 2000). *Populus tremuloides* Michaux clones can exhibit as much as a 10-fold variation in levels of foliar PGS across landscapes of North America (Lindroth and Hwang 1996; Donaldson et al. 2006b), which is of greater variation than that observed for primary metabolites (Lindroth 2011). This suggests that *Populus* species may have more diverse evolutionary strategies for defense than primary metabolism (Lindroth and St. Clair 2013).

Developmentally, PGs decline dramatically during the first ten years of life for *Populus* ramets (Donaldson et al. 2006a; Smith et al. 2011) but can revert back to the juvenile chemical profile of PGs after being cut back (Stevens et al. 2012), suggesting that many PGs may actually be targeting mammalian herbivores. Genotype largely dictates PG concentration in leaves and their impact on gypsy moth biomass and development (Osier and Lindroth 2006). However, environmental conditions, such as

high light, high carbon dioxide, and low nutrient availability can also increase concentrations of PGs (Lindroth et al.1993; Osier and Lindroth 2006). As previously mentioned, chemical defenses come at a cost, which in certain environments can reduce plant fitness. Osier and Lindroth (2006) observed growth of young saplings was inversely correlated with phenolic glycoside concentrations when the environment had low light and nutrient availability but was not correlated when all available resources were abundant. Older saplings showed the opposite effect, where PG production was more costly under high nutrient conditions, suggesting that older trees will devote more energy to growth once established (Stevens et al. 2007). The last two studies in particular, highlight how the interplay of genetics, environment, and development impact the production of PGs in *Populus*.

PGs may function as more reactive products following insect consumption (Clausen et al. 1990) by creating oxidative stress or binding covalently with proteins to hinder enzymatic reactions and disrupt nutrient uptake (Felton et al. 1992; Appel 1993; Summers and Felton 1994). PGs have been shown to be effective at reducing tree defoliation at low to moderate insect densities (Donaldson and Lindroth 2007) but at high densities where all leaf material is consumed these compounds have little effect (Donaldson and Lindroth 2007). Constitutive levels of PGs, such as salicortin and tremulacin, cause shifts in feeding preferences and distributional patterns, reduce consumption, and negatively impact growth and development in gypsy moth and FTC (Lindroth and Hwang 1996; Lindroth and Kinney 1998; Osier et al. 2000; Hemming and Lindroth 2000; Osier and Lindroth 2001; Osier and Lindroth 2004). As an example, Hemming and Lindroth (2000) fed fourth instar FTC and gypsy moth leaves

supplemented with 0, 2, or 4% of PGs and 0 or 5% casein. At the 4% PG concentration, there was an increase in duration in the fourth instar of FTC and gypsy moth (16 and 12% respectively) as well as a reduction in growth (27% gypsy moth, 14% FTC). Digestibility was also slightly reduced with increased PGs for gypsy moths but not for FTC (Hemming and Lindroth 2000). Numerous studies have shown levels of PGs within *Populus* can also shape the community of FTC and gypsy moth larvae populations (Lindroth and Hemming 1990; Lindroth and Bloomer 1991; Lindroth et al. 1991; Hemming and Lindroth 1995; Hwang and Lindroth 1997; Donaldson and Lindroth 2007). Previous herbivory damage may also change the chemical composition of PGs, although the only study demonstrating this effect occurred when substantial damage was done to young growing trees and PGs increased in new leaves produced right after the event (Stevens and Lindroth 2005).

While PGs are considered a constitutive defense, it is important to note that they may also be inducible (Clausen et al. 1989; Lindroth and Kinney 1998). Clausen et al. (1989) simulated herbivory by crushing of leaf tissue which resulted in significant increases in salicortin and tremulacin in leaves within 24 hours. The conversion of salicortin and tremulacin to 6-hydroxy-2-clyclohexenone and the degradation of these products to catechol, which are toxic to the large aspen tortrix, provided a plausible mechanism for short-term resistance in *Populus tremuloides* to insect herbivory. Lindroth and Kinney (1998) also demonstrated that gypsy moth defoliation induced PGs in *Populus tremuloides* (Michaux) aspen.

Induced Defenses

Probably the best-studied induced chemical defenses in *Populus* are a group of plant phenols called tannins. Tannins can be broken down into hydolyzable tannins (HT) and condensed tannins (CT) (Hagerman and Butler 1989). HTs contain structures with a core, usually glucose, which is esterified with galloyl groups (Barbehenn and Constabel 2011). CTs are polymers of flavan-3-ol subunits with chain lengths that can vary among *Populus* species (Schweitzer et al. 2008). Tannins are widely distributed in the plant kingdom (Mole 1993; Porter 1988) and found within various tissues of woody plants. Concentrations of tannins within plant species can be highly variable due to genotype, tissue development stage and environment. Levels of CTs in *Populus* leaves can be up to 25% of leaf dry weight (Donaldson and Lindroth 2008) but are generally much lower in woody tissues (Lindroth et al. 2007). CTs generally accumulate in the vacuole of leaves and fruit (Kao et al. 2002; Lees et al. 1993) while HTs are known to be concentrated in the cell wall of mesophyll cells (Grundhofer et al. 2001). In *P. tremuloides* and *P. angustifolia*, CT levels are twice as high in mature leaves as developing leaves (Donaldson et al. 2006b; Rehill et al. 2006). However, unlike PGs, CTs generally show an increase in levels during the first ten years of life for *Populus* (Donaldson et al. 2006b). Increases in CT as trees mature suggest that those compounds are targeting insect herbivores (Lindroth and St. Clair 2013). There is some debate as to whether CT have a role in IR in *Populus* to common lepidopteran pests. To date there is little evidence that supports the role of CTs as defenses against major lepidopteran pests of *Populus* (Osier and Lindroth 2001; Osier and Lindroth 2004; Schweitzer et al. 2008; Constabel and Lindroth 2010; Lindroth and St. Clair 2013). CT genes do appear to be induced upon wounding and herbivory (Osier and Lindroth 2001; Kao et al. 2002; Peters and Constabel

2002; Tsai et al. 2006). For example, dihydroflavanol reductase enzymes are involved in CT synthesis and is significantly induced 24h following mechanical wounding feeding by satin moth larvae (*Leucoma salicis* Linnaeus) and FTC (Peters and Constabel 2002). In other tree species tannins can play a role in IR. For example, Kopper et al. (2002) reported that WMTM larve reared on a diet with higher CT concentrations from paper birch *Betula papyrifera* Marshall had increased instar duration, decreased relative growth rate, and decreased food conversion. However, WMTM ate more and grew larger on diets containing tannin than the control diet. While there is limited evidence support to a direct role in IR in *Populus* tannins, they may facilitate rapid nutrient uptake and plant recovery following herbivory (Lindroth and St. Clair 2013) and may protect the tree from pathogens such as shoot blight (Holeski et al. 2009). Also, *Populus tremuloides* (Michaux) tannins are negatively correlated with performance of specialized aspen-feeding chryosomelids (Donaldson and Lindroth 2004).

Some proteinaceous defenses, such as the kunitz protease inhibitors (KPI) and chitinases, are induced in response to wounding and insect herbivory. Protease inhibitors may have roles in regulating endogenous protease activities, storage proteins, or biochemical defenses (Ryan 1973; Richardson 1977; Phillipe et al. 2009). KPI's are induced by insect feeding or mechanical wounding in a number of studies (Bradshaw et al. 1990, Haruta et al. 2001, Christopher et al. 2004; Ralph et al. 2006; Philippe and Bohlman 2007). There is also evidence from *in vitro* studies that KPI's directly inhibit proteases in FTC gut extracts (Major and Constabel 2008). A study by Philippe et al. (2009) identified 31 KPIs in the *Populus trichocarpa* genome. There are six *Populus* specific subfamilies of KPIs, suggesting that tandem duplications are driving the

expansion of these protease inhibitors. There is not a lot of evidence that KPI's are effective against insect herbivory in *Populus*, but when the win3-encoded KPI protein from *Populus* was expressed in tobacco and tomato it lead to decreased larval weight gain of tobacco budworm (*Heliothis virescens* Fabricius) (Lawrence and Novak 2001).

Chitinases were among the first defensive genes found in *Populus*. Chitinases are glycosyl hydrolases that can degrade chitin to low molecular weight chitooligomers (Hamid et al. 2013). It is thought that plant chitinases may target the peritrophic membrane of the insect gut, which contains chitin as a protective barrier for the insect gut (Richards and Richards 1977; Chapman 1985). *Win6* and *win8*, basic chitinases, are strongly and systemically induced in *Populus* in response to wounding (Parsons et al. 1989; Davis et al. 1991; Clarke et al. 1998). Jiang et al. (2013) identified 37 chitinases with a complete open reading frame in *Populus trichocarpa* that could be grouped into five different classes of chitinases using phylogenetics. As seen with the KPIs, some chitinase subfamilies, such as the class 1 and class 3 chitinases, were in localized tandem repeats. Within chitinase families some genes were highly expressed when exposed to fungus, methyl jasmonate, and following wounding (Jiang et al. 2013). The best evidence for their role in defense against insects comes from a study by Lawrence and Novak (2006). Colorado potato beetles that fed on transgenic tomato plants with the *win6* chitinase from *P. trichocarpa x P. deltoides* Bartr. showed slower development compared to control plants.

Several other studies have been done to explore inducible genes and their role in *Populus* defense against insect herbivory through indirect means. These studies have identified signaling compounds, such as methyl jasmonate (Wu et al. 2008) and ethylene

(Benavente and Alonso 2006), and volatile organic terpenoids used in tritrophic interactions intended to attract enemies of herbivores (Arimura et al. 2004). In many plant species herbivore induced volatiles act as direct defenses because of their toxicity or as indirect defenses by attracting natural enemies of the herbivore (Yuan et al. 2008). Volatile emissions of mono-, sesqui-, and homo-terpenoids, phenolics, and benzene cyanide appear to be herbivore induced in *Populus* as an indirect defense to attract parasitoids (Ariumura et al. 2004). Terpenoids can be increased by the cytosol-localized mevalonate (MVA) pathway or the plastid-localized methylerythritolphosphate (MEP) pathway (Tholl 2006). Formation of monoterpenes, diterpenes, and sesquiterpenes is catalyzed by terpene synthases (Chen et al. 2009). The terpene synthase gene family consists of 38 members within *Populus trichocarpa* (Irmish et al. 2014). They were able to identify and isolate 11 terpene synthase genes from gypsy moth damaged leaves. A subset of these genes were found to be upregulated after herbivory and to be regulated by jasmonates. Interestingly, gene expression differences were observed in damaged leaves only, suggesting no systemic induction. Sesquiterpenes have been shown to be released due to insect herbivory, such as (*E,E*)-α farnescene from FTC damage (Arimura et al. 2004) and (*E*)-β-caryophyllene from gypsy moths (Frost et al. 2007). McCormick et al. (2014) tested volatile emission from damaged and adjacent undamaged leaves of *Populus nigra* after herbivory by gypsy moth caterpillars to determine whether compounds attracted the gypsy moth parasitoid *Glyptapanteles liparidis* (Braconidae). Volatiles released by damaged leaves included terpenes, green leaf volatiles, and nitrogen containing compounds such as aldoximes and nitriles. Volatile emission release appeared to be regulated by jasmonate signaling and local activation of volatile biosynthesis.

Further work needs to be done to identify and characterize genes involved in indirect defenses and to identify volatiles that are involved in recruitment of lepidopteran parasitoids.

Significant research has been done to identify genes with transcriptional responses to insect herbivory. Christopher et al. (2004) used macroarrays with 580 cDNAs to look at leaf responses 24 hours after mechanical wounding or wounding with FTC regurgitant in *P. trichocarpa x P. deltoides.* They observed that chitinases, PPO, KPIs, vegetative storage proteins, and genes involved in octadecanoid and phenylpropanoid biosynthesis were induced by both treatments, but the magnitude of induction was generally larger in the wounding plus regurgitant treatment. Lawrence et al. (2006) used RNA differential display to identify 57 distinct genes that were differentially expressed in leaves after feeding by gypsy moth or after mechanical wounding compared to control leaves. They identified three sequence motifs in the 5' region of 15 of the wound-induced genes that were overrepresented (i.e., DRE element, W box, and H box), potentially identifying regulators of wound-inducible promoters. Major and Constabel (2006) used a macroarray with 580 cDNAs to compare the transcriptome profiles of *P. trichocarpa x P. deltoides* elicited by wounding and regurgitant from FTC in treated and systemic leaves. They identified wound-responsive genes involved in primary and secondary metabolism and found three unknown genes with a ZIM motif that may be transcription factors. These proteins were later called jasmonate ZIM-domain (JAZ) proteins and they are important regulators of jasmonate signaling in that they repress transcription of genes that respond to jasmonate (Thines et al. 2007, Chini et al. 2007). Overlapping expression was observed in directly treated and systemically induced leaves. The chemical volicitin from

insect regurgitant was identified as the factor that induced expression of these genes. Ralph et al. (2006) used a microarray with 15,496 cDNAs to measure changes in gene expression after 24 hours of FTC feeding. They identified 1,191 up-regulated genes and 557 down-regulated genes in response to insect feeding. Induced defenses included proteinaceous defenses, like KPI's and chitinases, enzymes involved in secondary metabolism (e.g. cytochrome P450s, polyphenol oxidase, terpene synthases), as well as many new genes that were previously not associated with plant defense. These included leucine-rich repeat containing transmembrane/receptor-like kinases, ATP-binding cassette (ABC) proteins, and genes involved in carotenoid biosynthesis and many different transcription factors. Miranda et al. (2007) also looked at the *P. trichocarpa x P. deltoides* leaf transcriptome in response to poplar leaf rust (*Melampsora medusae*) as well as FTC feeding. Many genes that were repressed by rust at 3, 6, and 9 days post-innoculation were induced after 24 hours of insect feeding. This suggests a possible antagonistic response of *Populus* to the pathogen versus insect feeding.

Babst et al (2009) used microarrays to examine the transcriptional response of *P. nigra* leaves to gypsy moth feeding or the stress hormone jasmonic acid in local and systemic leaves. Eight hundred genes were induced, although only 14% of those were induced in response to both gypsy moth herbivory and exogenous jasmonic acid. This suggests that although jasmonates are released in response to insect feeding, it is not a good treatment to identify genes involved in the response to insect herbivory. Gypsy moth feeding resulted in the up-regulation of genes involved in jasmonate biosynthesis, abscisic acid biosynthesis, and other signaling pathways. Babst et al. (2009) also observed that gypsy moth feeding on local leaves resulted in greater gene expression

differences than untreated systemic leaves and genes that did respond in systemic leaves were a subset of those found in local leaves. The authors suggested that genes induced in local and systemic leaves may be the highest quality IR candidates for additional future work.

Philippe et al. (2010) measured transcriptional responses in local source, systemic source, and systemic sink leaves 2, 6, and 24h following the application of FTC oral secretions on mechanically wounded leaves. Genes with the greatest expression differences between local source and systemic sink leaves included galactinol synthase, heat shock proteins, KPIs, and proteins of unknown functions. Phillippe et al. (2010) also observed rapid and strong expression differences in local source leaves, weaker and slower responses in systemic source leaves, and faster stronger responses in systemic sink leaves (compared to systemic source leaves). While a significant number of genes have been collectively identified as potential targets in these studies, future transcriptomic studies in *Populus* should look at 1) induction of gene expression in response to different insects, 2) temporal patterns of activated defensive genes, and 3) earlier time-points in order to capture early molecular events that initiate in defensive signaling cascades (Ralph 2009).

Other important studies have focused on validating targets identified from these microarray experiments to confirm their role in IR. Biochemical characterization has been done via heterologous expression of recombinant proteins in yeast or bacteria. On addition, purified protein or metabolite has been added to an artificial diet to study their impacts on insect growth and development (Major and Constabel 2008; Arimura et al. 2004; Peters and Constabel 2002). Transgenics have also been a successful approach to

study herbivory-induced genes and their effectiveness in inhibiting insect performance. McCown et al. (1991) generated *P. alba x P. grandidentata* and *P. nigra x P. trichocarpa* transgenic lines to express d-endotoxin that negatively impacted feeding of FTC and gypsy moth larvae. Gill et al. (2003) used transgenic *P. tremula x P. alba* INRA 717-1-B4 to over-express tryptophan decarboxylase. Tryptophan decarboxylase catalyzes the decarboxylation of tryptophan to tryptamine, which may inhibit insect development and reproduction. The transgenic *Populus* were shown to have higher levels of tryptamine, which resulted in reduced leaf consumption by FTCs feeding on control trees.

Conclusion

Substantial work has been made done to understand how plants, specifically *Populus*, defend themselves against lepidopteran pests. We now have comprehensive inventories of genes that are transcriptionally activated and repressed in response to insect herbivory, as well as a comprehensive list of secondary metabolites involved. What remains to be determined is which genes are regulating the defense response. With the development of AT *Populus* mutant lines we can now use forward genetics as a complimentary approach to continue to identify genes and gene regulatory networks involved in IR. Screening of AT *Populus* by Ralph (2009) for mutants with altered resistance to WMTM feeding has identified several mutants for further validation with bioassays. The next chapter will discuss forward genetics, AT, and discuss how we screened QU and MTU AT mutants to identify mutants with altered resistance to WMTM.

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CHAPTER III

BIOASSAY SCREENING OF ACTIVATION TAGGED *POPULUS* TO IDENTIFY MUTANTS WITH ALTERED INSECT RESISTANCE

Contributions of Authors

QU Experiments

AT *Populus* were created by Dr. Sharon Regan at Queen's University. Dr. Steven Ralph propagated QU candidates from stem cuttings with assistance from Justin Burum, Haylee Dassinger, Alicia Grant, and Diana Bertrand. Justin Burum performed air-layer propagation, choice bioassays, and no-choice development bioassays for QU candidates with assistance from Alicia Grant and Haylee Dassinger.

MTU Experiments

AT *Populus* were created by Dr. Victor Busov at Michigan Technological University. Dr. Steven Ralph propagated mutant lines in tissue culture and maintained healthy trees in incubators at UND with assistance from Justin Burum, Samuel Bandi, Heidi Connahs, Brett Gross, Alicia Grant, and Aubree Wilke. Insect care and regurgitant collection was primarily done by Justin Burum, with assistance from Heidi Connahs and Samuel Bandi. Justin Burum screened 392 mutants (50.9%) with assistance from Brett Gross, Aubree Wilke, and Eric Jagim. Samuel Bandi, Heidi Connahs, and Steven Ralph screened the remaining mutant pairs. Justin Burum conducted choice bioassays

comparing mutant candidates against *Pt x Pa* wildtype. Histograms for each choice bioassay, comparison of leaf area FPR and leaf weight FPR, and graphs for individual mutant-wildtype comparisons with statistical analysis were done by Justin Burum.

Introduction

Despite the knowledge that has been gained from studies highlighted in Chapter 2, regulatory mechanisms of many plant defensive pathways are still unknown. Prior work in *Populus* to validate genes of interest in response to insect herbivory involved a reverse genetics approach. Experiments were conducted to generate lists of genes impacted by herbivory and candidates were followed up by creating transgenics to confirm their role in IR. While this approach has been successful, it has also revealed that genes previously hypothesized to be involved in defense may not actively contribute to IR. Two examples of genes that have been shown to be induced after insect feeding but have not been shown to be involved in IR in *Populus* are polyphenol oxidase (PPO) and ascorbate oxidase (AO) in *Populus*. PPOs catalyze the oxidation of ortho-diphenolic compounds to quinones (Vaughn and Duke 1984). One proposed IR mechanism for PPO is that quinones cross-link proteins and amino acids during feeding, decreasing the insect's ability to absorb amino acids (Duffy and Felton 1991). To test whether or not PPO causes IR Wang and Constabel (2004) created transgenic poplar to significantly over-express PPO and tested them on different insect species. They determined that only first instar FTC larvae from older egg masses showed higher mortality and reduced weight gain when fed on transgenic leaf disks as compared to wildtype leaf disks. Insect feeding choice was no different for wildtype versus mutants. Barbehenn et al. (2007) also used up-regulated PPO Populus to examine effects on consumption and growth rate of

gypsy moth and WMTM. There was no difference in either consumption or growth rate between insects that fed on mutant versus wildtype leaf disks, suggesting that more controlled mechanistic studies need to be done on plant-herbivore systems to determine if PPO has an effect on insect performance.

Ascorbate oxidase (AO) has also been investigated for its potential role in poplar defense. Ascorbate is an antioxidant that is an essential nutrient involved in catalyzing the oxidation of L-ascorbate to dehydroascorbate in insects (Barbehenn et al. 2008) and has been shown to be induced in response to insect herbivory and wounding (Felton and Summers 1993; Bi et al. 1997). Barbehenn et al. (2008) used transgenic *Populus* with 14-37 fold higher foliar expression of AO than wildtype plants to examine effects on gypsy moth and *Melanoplus sanguinipes* grasshoppers. No significant difference in ascorbyl radicals within the insect gut or relative consumption rate, growth rate, or nutritional indices between transgenic and wildtype trees were found. The authors of this study concluded that AO is unlikely to serve as a defense against these two insect species as there was no impact on feeding and development.

These examples highlight that induction of genes following insect herbivory does not always correlate to an active role in defending the plant. An alternative or complimentary approach that can be applied to identifying genes involved in plant defense is forward genetics. Forward genetics, specifically using activation tagging (AT) in *Populus*, may reveal genes that can influence plant defense from leaf defoliating insects.

Forward Genetics

Forward genetics has become a successful approach to identify genes responsible for a trait of interest in plants (Alonso and Ecker 2006). Forward genetics begins with the generation of a randomly mutated population, screening that population for alterations in the phenotype of interest, and then identifying the mutated gene that is causing the resulting phenotype (Alonso and Ecker 2006). One *Agrobacterium* T-DNA insertional mutagenesis approach that has been successfully applied in a variety of plant species, including *Populus*, has been AT.

In nature, *Agrobacterium tumefaciens* is a soil bacterium responsible for crown gall disease in over 140 plant species. To infect the plant, *Agrobacterium tumefasciens* inserts a tumor-inducing plasmid. The plasmid contains a small segment of DNA, called a transfer DNA (T-DNA), as well as several additional genes necessary to transfer the plasmid into the plant host cell, cut out the T-DNA, and randomly integrate the T-DNA in actively transcribed regions of chromosomal DNA of the plant cell. The T-DNA encodes genes to produce auxin or indole-3-acetic acid that stimulate cell proliferation and the formation of galls. In AT, the T-DNA is modified to remove genes that induce gall formations. These genes are replaced with a series of strong enhancers positioned next to the right or left hand border of the plasmid. The T-DNA inserts randomly within the plant genome through the use of *Agrobacterium tumefasciens* and the enhancers can recruit transcription factors to the promoter region of nearby genes to produce gain of function mutants (Weigel et al. 2000).

A few decades ago Walden and colleagues (Hayashi et al. 1992) constructed the first T-DNA vector with four copies of an enhancer element from the cauliflower mosaic virus (CaMV) 35S gene and used *Agrobacterium tumefaciens* to infect the plant (figure

5). The AT mutants containing the T-DNA insert showed the same tissue/cell pattern of gene expression in wild-type plants, but the level of expression was enhanced approximately 5-10 fold. Altered phenotypes in mutants were related to normal endogenous gene function but at a higher level of transcription than in wildtype plants. Since that initial study a number of different AT vectors have been used to look at a variety of phenotypes including flowering (Lee et al. 2000; Kardailsky et al. 1999), secondary metabolism (Woodward et al. 2005; Borevitz et al. 2000), growth and development (Busov et al. 2003; Zhang et al. 2006; Schomburg et al. 2003; Li et al. 2002), abiotic stress (Yu et al. 2008; Zhang et al. 2008; Yoo et al. 2007), pathogen resistance (Koch et al. 2006; Xia et al. 2004; Grant et al. 2003; Suzuki et al. 2004) and IR (Ralph 2009; Chen et al. 2012).

Activation Tagging in *Populus*

AT is ideally suited for *Populus* because: 1) mutants express a dominant phenotype that is typically visible in the primary transformant (important in *Populus* that do not develop flowers for 7-10 years) and 2) the frequency of visible mutants observed in screens of *Populus* is high compared to *Arabidopsis*, likely due to a high level of epigenetic gene silencing in *Arabidopsis* in second generation progeny (Harrison et al. 2007). Dr. Steve Strauss's lab described the first AT *Populus* population (Busov et al. 2003) where 627 independent mutant lines were generated using *Agrobacterium tumefasciens* to insert the AT vectors pSKI015 and pSKI074 into the *Pt x Pa* genetic background. Transformants were screened for visible phenotypes in tissue culture, greenhouse, and field environments. The first phenotype identified was a dwarf variety with reduced internode length and overall stature that was caused by overexpression of

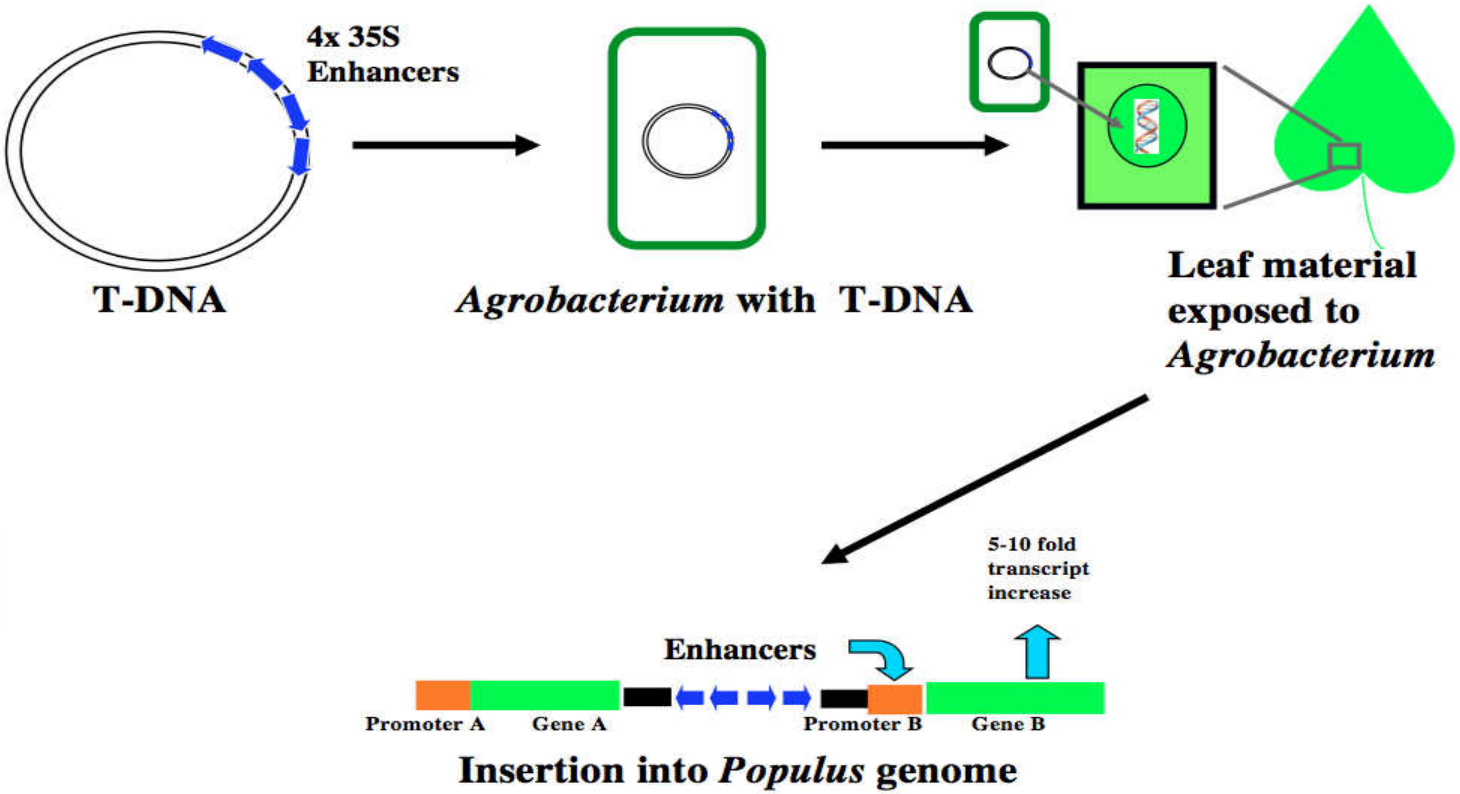

Figure 5: Activation tagging procedure. A T-DNA, containing four copies of the CaMV enhancer is transformed into *Agrobacterium tumefasciens*. Leaf tissue is inoculated with *Agrobacterium* which randomly inserts the T-DNA within the *Populus* genome, increasing transcription of a nearby gene by enhancing its promoter. AT mutants can then be grown in tissue culture and then transferred to the greenhouse to be screened for phenotypic differences.

GA 2-oxidase gene (Busov et al. 2003). Since then a few populations have been created and screened for various traits. Dr. Sharon Regan at QU has created the largest population to date using *Agrobacterium* mediated transformation with the pSKI074 AT vector (1,800 trees; Harrison et al. 2007). Phenotypic analysis of greenhouse-grown plants in the QU population has assessed leaf size, leaf shape, leaf texture, leaf spacing, stem size, stem shape, stem texture, internal wood color, overall size, and tree shape. Using these features, at least 50 different visible phenotypes have been observed among the 1,800 lines, indicating an altered phenotype rate of about 3% (Harrison et al. 2007).

In a study by Busov et al. (2010) 627 independent AT events were planted outdoors in a two-year field study. Prior to the field exposure 1.6% of AT *Populus* showed a phenotypic difference under greenhouse conditions. However, after two growing seasons in the field nearly 6.5% of the mutants showed visable phenotypes. Therfore, AT *Populus* allowed to interact with the environment revealed a greater number of mutants with detectable phenotypic differences.

A few interesting visible phenotypes have been identified from these AT populations. One mutant identified from the AT populations from Harrison et al. (2007) and Busov et al. (2010) showed enhanced woody growth and changes in bark texture caused by increased secondary phloem production (Yordanov et al. 2010). The mutation was identified as an activation of a novel lateral boundaries domain (LBD) transcription factor (Yordanov et al. 2010). Suppression of the gene lead to decreased diameter growth and irregular phloem development. In a study by Trupiano et al. (2013) they identified five mutant AT lines with changes in adventitious rooting in *Populus*. In one line with

increased adventitious rooting they identified the up-regulated gene as an AP2/ERF transcription factor.

These studies highlight how AT mutagenesis has been successfully used to identify genes involved in morphological phenotypes. IR is, however, not a visible phenotype and cannot be determined simply by looking at the plant. The IR phenotype can only be observed in response to insect feeding. Therefore screening needs to be conducted on many AT mutants in a manner that can measure IR to identify candidates for eventual T-DNA mapping and identification of genes responsible for the phenotype (Ralph 2009).

One approach to identify genotypes with IR has been to conduct molecular genetic screening for specific plant metabolites known to increase plant IR, followed by insect bioassay validation and identification of mutation(s) contributing to the phenotype. An example of this approach involved screening for leaf glucosinolates in *Arabidopsis.* Beginning with bioassays to identify leaf glucosinolate content based on quinone reductase activity in *Arabidopsis* (Gross et al. 2000), Grubb et al. (2002) were able to positively correlate quinone reductase and leaf glucosinolate profiles using leaf disks. Cell-free leaf extracts from a large collection of AT *Arabidopsis* mutants were screened for altered quinone reductase activity (Wang et al. 2002). One gene identified to have an altered glucosinolate profile, later called *IQD1,* was shown to be induced by mechanical wounding of leaves and after infestation of peach aphids (Levy et al. 2005). This approach has also been used to identify *Arabidopsis* AT genotypes with increased phenolics. Gigolashvili et al. (2007) they identified a mutant, *HIG1*, with increased expression of an R2R3 MYB transcription factor that activated indolic glucosinolate

genes. This type of approach allows rapid screening for specific metabolites associated with the phenotype of interest. The main drawback of this strategy is that plant defenses not related to altered metabolite profiles will not be detected. Furthermore, performing a comprehensive metabolite profile to identify statistically significant differences is costly and time consuming.

An alternative strategy is to conduct greenhouse, laboratory, or even field studies using whole or parts of the plant to directly assess plant defense. A study by Chen et al. (2012) screened an AT collection of *Arabidopsis thaliana* using the green peach aphid *Myzus persicae* (Sulzer) and the virus *Turnip yellows virus* (*TuYV*). AT lines were exposed to peach aphids carrying the virus and then screened for the efficiency of virus transmission as an indicator of insect resistance. Mutants that were virus free following aphid feeding were characterized and showed increased resistance to aphids, suggesting that their approach allows for rapid screening of thousands of mutants (Chen et al. 2012). This work has lead to the identification and characterization of several different mutants. In 2013 Chen et al. characterized one of the AT aphid resistant mutants and identified the activated gene as *At5g65040*, later renamed *Increased Resistance to Myzus persicae* 1 (*IRM1*). New transgenics over-expressing *IRM1* resulted in aphid resistance, confirming observations from the original AT mutant. Using an electrical penetration graph, this mutant had an enhanced mechanical barrier to prevent aphids from reaching the phloem. On the other hand, aphids easily penetrated the plant tissue of knock-out mutants and more rapidly penatrated through the xylem to the phloem.

In 2014 Chen et al. characterized another aphid resistant mutant with over-expression of *SKS13*, a gene normally expressed only in pollen and non-responsive to

aphid attack. *SKS13* over-expression resulted in aphids having difficulty feeding on leaves. Accumulation of reactive oxygen species in this mutant may be regulated by the jasmonic acid pathway and cause the IR phenotype (Chen et al. 2014). While there are not many examples of bioassays approach being used to screen mutagenized plant populations, this strategy has been employed to screen cultivars using either detached leaves or whole plants in choice and no-choice assays for IR (Sharma et al. 2005; Shankar et al. 2014; Nordman et al. 2005). Within the last ten years a few populations of AT *Populus* mutants have been generated and screened for mutants with morphological abnormalities (Busov et al. 2003; Harrison et al. 2007). However, screening for IR has been limited (Ralph 2009). Our goal was to use these AT *Populus* populations and bioassays to identify genes involved in IR.

Proof of Principle of Using Bioassays to Detect IR

Before a large screen in *Populus* was attempted, bioassays were conducted on the AT mutant *fuzzy* (Plett et al. 2010) to prove it was possible to identify IR mutants. The *fuzzy* mutant was initially identified from a screen of the AT QU population for having a visible "fuzzy" leaf texture due to greater trichome density (Harrison et al. 2007). Characterization of the mutant later confirmed that the increased trichome density was caused by over-expression of *MYB186* (Plett et al. 2010). Plett and colleages from the Ralph lab used three different bioassays to test the hypothesis that WMTM larvae feeding on *fuzzy* would exhibit reduced consumption and retarded development compared to larvae feeding on wild-type leaves.

A choice bioassay was conducted to measure leaf consumption when WMTM larvae were given both wildtype and *fuzzy* leaf disks from unwounded leaves. This

bioassay determined whether WMTM had a preference for feeding on mutant or wildtype leaves. Leaves were harvested and cut into disks from *fuzzy* and wild type trees using a copper pipe, and then pinned in an alternating pattern on large Petri dishes with wet paper towels. Following 24 hours of starvation, four fourth-instar WMTM larvae were allowed to feed on the leaf disks for 24 hours. Remains of leaf disks were scanned and analyzed using a custom-modified imaging program GNU Image Manipulation Program (GIMP) version 2.4.6 (http://gimp.org) to calculate leaf area remaining.

The second bioassay performed was a no-choice development bioassay in petri dishes. The purpose of this bioassay was to measure WMTM leaf consumption, wet weight gain, and development (instar stage) when larve were given leaf material from either wildtype or *fuzzy* leaf disks over a two-week period. Leaf consumption and insect development could be accurately measured when newly hatched larvae were confined to petri dishes. LPI 11-13 leaves were harvested and cut into leaf disks. Wildtype or *fuzzy* disks were pinned on wet paper towels in small plates. Eight first instar larvae, three days post hatch, were placed on each plate and allowed to feed. Every two days the leaf area remaining from each plate was scanned and analyzed using GIMP. New leaf disks were cut and placed in petri plates from freshly harvested leaves. Insect development was monitored every two days and insect weight gain was measure on days six, ten, and 14.

The third bioassay was an on-tree no-choice development bioassay. Like the petri-plate no-choice development bioassay, this bioassay was designed to measure WMTM larvae weight gain and development when reared on leaf material from either *fuzzy* or wildtype trees over a longer period of time (7-12 days). However, instead of cutting leaf disks from trees, late second instar WMTM larvae were caged with nylon mesh bags

around 10 leaves of the same LPI stage on either fuzzy or wildtype trees. This type of bioassay allowed a more realistic evaluation of the effect of the tree on WMTM larvae.

Choice feeding bioassays, conducted to measure leaf consumption after 24 hours by 3rd and 4th instar larvae, found that larvae fed significantly less on *fuzzy* compared to wildtype leaf disks (Plett et al. 2010). Petri plate no-choice development bioassays revealed that WMTM larvae raised on wild-type leaf discs in Petri dishes developed through 4th or 5th instar and gained, on average, 20.9 mg in fresh weight whereas larvae reared on a diet of *fuzzy* leaf discs only developed to the 3rd or 4th larval instar and gained only 5.8 mg (Plett et al. 2010). In the on-tree no choice development bioassay, larvae caged with nylon mesh on wildtype trees grew significantly larger than larve caged on *fuzzy* trees(Plett et al. 2010*)*. The three bioassays confirmed that WMTM preferred consuming wildtype leaves over *fuzzy* leaves and larvae displayed reduced development when raised on *fuzzy* leaves. The study also showed that bioassays results could be used to identify IR AT *Populus* mutants.

AT and *Populus* IR

The first screen for altered IR in AT *Populus* was conducted by using choice bioassays on 608 mutants from the QU population (Ralph 2009). Unwounded leaves from the same LPI for two different AT mutants were cut into leaf disks and then pinned in alternating pattern on wet paper towels in large petri plates. After 24 hours of starvation, five or six third and fourth WMTM instar were placed on each plate and allowed to feed for 24 hours. Areas remaining for leaf disks from each mutant were measured using GIMP and a feeding preference ratio was calculated by dividing the area remaining from one mutant over the other mutant in each pair (Ralph 2009). The screen

identified 22 pairs as having a feeding preference ratio greater than 1.5 standard deviations from the population mean. These mutant pairs suggested that at least one mutant within each pair was either a resistant or sensitive candidate for further testing (Ralph 2009). The screen ultimately showed that choice bioassays could be used to identify IR candidates in the AT mutant population. The screen revealed several interesting mutants for further validation and characterization, setting up for the work conducted in this dissertation.

The purpose of any forward genetics screen is to be quick and qualitative so that more energy can be devoted toward testing promising candidates. To rapidly screen the AT population, mutant-mutant comparisons using a single biological replicate in choice bioassays was performed at QU (Ralph 2009). Mutant-mutant comparisons were not viewed as a problem as previous greenhouse studies only identified up to 3% of the population with altered visable developmental phenotypes (Harrison et al. 2007). If IR follows the same mutation rate as visible phenotype mutations in AT *Populus*, then we would estimate that at least 97% of mutants would not have an altered IR phenotype. The use of a single biological replicate for each mutant in choice bioassays can increase the odds of false positives among the candidate pairs. Also, mutant-mutant pairs where insects had a substantial feeding preference for one mutant over the other would require additional bioassays to establish which mutant(s) is the candidate to pursue. To overcome the biological and mutant-mutant comparison limitations from the initial screening effort, additional validation of mutant candidates using bioassays against a wildtype background with greater biological replication would be required. In addition, choice bioassays only determined if insects had a preference for one mutant versus another. It does not

necessarily mean that the preference will result in an impact on growth and development of the insect. Additional no-choice assays for mutants identified from the screen would need to be used to determine if the mutant has an effect on the insect.

Objective #1 of Dissertation

Objective #1 of this dissertation was to identify AT *Populus* mutants with altered IR to WMTM using choice and no-choice bioassays. To achieve this objective candidates identified from the QU screen were brought back to UND, propagated, and tested against wild-type trees using choice bioassays with extensive biological and technical replication. Of the nine mutants screened, only one mutant, *E8-16* consistently and substantially affected WMTM behavior, with larvae consuming more from *Pt x Pa* wildtype leaves than mutant leaves. *E8-16* underwent further testing to assess its impact on insect growth and development in no-choice on-tree and no-choice petri plate development assays. We also screened an additional 770 AT *Populus* mutants created by Dr. Victor Busov from MTU to find additional candidates. In this new screen we expanded the original bioassay design by using choice bioassays with unwounded and wounded leaves. This new screen was a novel approach to identify mutants that have altered constitutive and induced defenses to WMTM larvae.

Methods

QU Unwounded Choice Bioassays

***Populus* propagation for bioassays.** Stem cuttings for nine candidates from the QU screen in 2009 (*E14-56*, *E7-4*, *E8-13*, *E8-16*, *EB25-9*, *R22-6*, *R1-1*, *B7-17*, and *R8-23*) were initially propagated in "cone-tainers" by making a fresh cut on the stem, applying Hormex rooting powder on the wound site, and placing the stems in a 3:1 mix

of Sunshine Mix #1 potting soil and sand. Stem cuttings were grown in an incubator at 25°C with 55-65% humidity until root development. As the saplings grew they were transferred to 1-gallon pots, and then later to 3-gallon pots. Candidate lines were further propagated using air-layering due to greater rooting and propagation success. Parent plants were grown with four lateral branches from the main stem. Branches were wounded by making an angular incision 1/3 to 1/2 of the way through the stem between nodes 10-15 cm from the branch apex. Incision sites were held open with toothpicks or large piece of sphagnum moss to prevent the branch from healing around the wound site. Rootone (GardenTech; Palatine, IL, USA) rooting hormone was applied to the wound site with a cotton swab to induce rapid root development. Damp sphagnum moss was placed around the stem at the wound site and wrapped in aluminium foil. Newly air-layered branches were monitored for four weeks, with water added to the sphagnum moss as needed. Newly rooted daughter plants were cut from the parent plant below the moss and potted in a 3:1 mix of Sunshine Mix#1 potting soil and sand, staked, and then covered with a clear plastic bag for three days. Bags were removed on cloudy days or at night to reduce plant stress. For pictures of the air-layering process see figure 6. Candidate lines and wildtype trees were propagated at the same time to produce 6-10 biological copies of the same age for each unwounded choice bioassay. All propagation and growing of trees occurred in the Starcher Hall greenhouse at the University of North Dakota (UND). Air-layering of QU AT lines occurred between March 2010 and May 2011 where greenhouse temperatures were 70-94°F. Trees were provided supplemental lighting to provide a constant 16:8 day/night cycle even in the winter and were watered and fertilized *ad libitum*. Trees were routinely inspected and sprayed with cold water to ensure trees

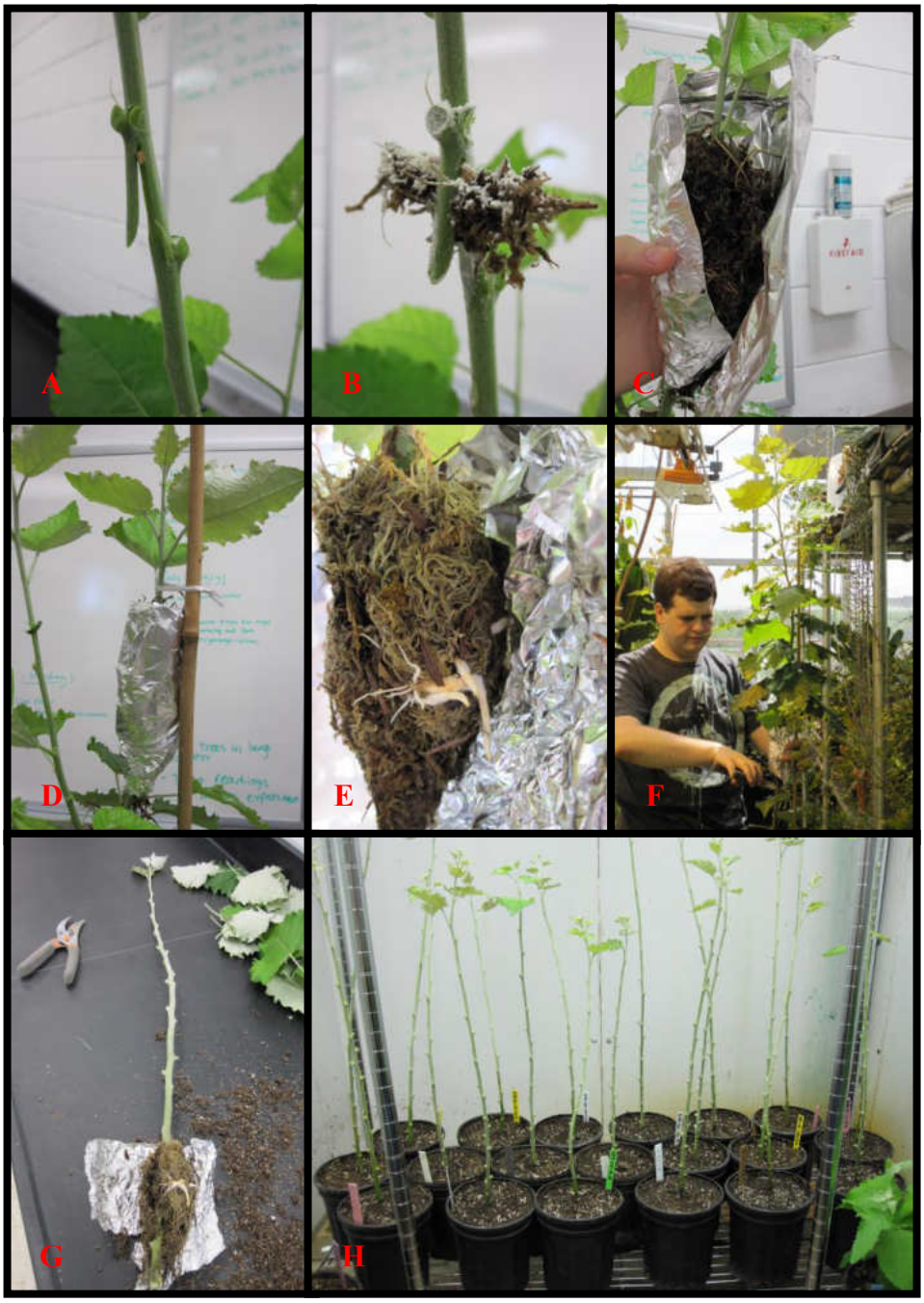

Figure 6: Propagation of AT *Populus* mutants using air-layering. A) Angular incisions were made using a razor blade on branches approximately 20 cm from the apex. Wound site was held open with toothpicks. B) Rooting hormone was applied to the incision site and nearby nodes to encourage root development. C) Damp sphagnum moss was placed around and within the incision site and wrapped with aluminum foil leaving the top open for watering. D) Air-layers were staked to encourage drainage of excess water. E-H) Four weeks following air-layer creation roots have formed. Air-layers were cut from the parent plant, potted in 1gallon pots, bagged for three days, and then gradually acclimated to the greenhouse.

remained healthy and spidermite free.

Insect propagation. All WMTM eggs were purchased from the Insect Production Service of the Canadian Forest Service. Insects were reared in a growth chamber (Percival Scientific model #166LVL; Perry, IA, USA) at 50% relative humidity with a 16:8 day-night cycle and 22:18ºC day/night temperatures on General Purpose Lepidopteran Diet with 14% aureomycin antibiotic (BioServ; Flemington, NJ, USA). Insects were reared to third and fourth instar larvae for choice assays and late second instar for on-tree no-choice development bioassays. Insects used for petri plate no-choice development bioassays were first instar, two days post hatch.

Unwounded choice assays. Procedures for unwounded choice bioassays were done as described in Wang & Constabel (2004) with some modifications. Six to ten air-layered biological copies of each AT candidate line, along with *Pt x Pa* wild-type biological replicates, were arranged in an alternating pattern on greenhouse benches and grown until they reached a height between 80-100 cm. Mutant and *Pt x Pa* wildtypebiological replicates for each comparison were phenotyped for height, number of leaves, and overall health and paired based on similar height and appearance. Leaves designated as LPI 11-13 (Larson and Isebrands 1971) were harvested by cutting the leaves off at the petiole for each mutant-wildtype pairing at the petiole using greenhouse clippers (figure 7). Leaves were briefly rinsed with water and gently dried with paper towels before 6.5 cm^2 leaf disks were cut using a sharpened copper pipe. Four disks from mutant and *Pt x Pa* wildtype trees were pinned in an alternating pattern on a wet paper towel with colored pins (figure 7). Three to four technical replicate plates were prepared for each biological pair. Ten additional leaf disks were cut, placed in brown paper bags,

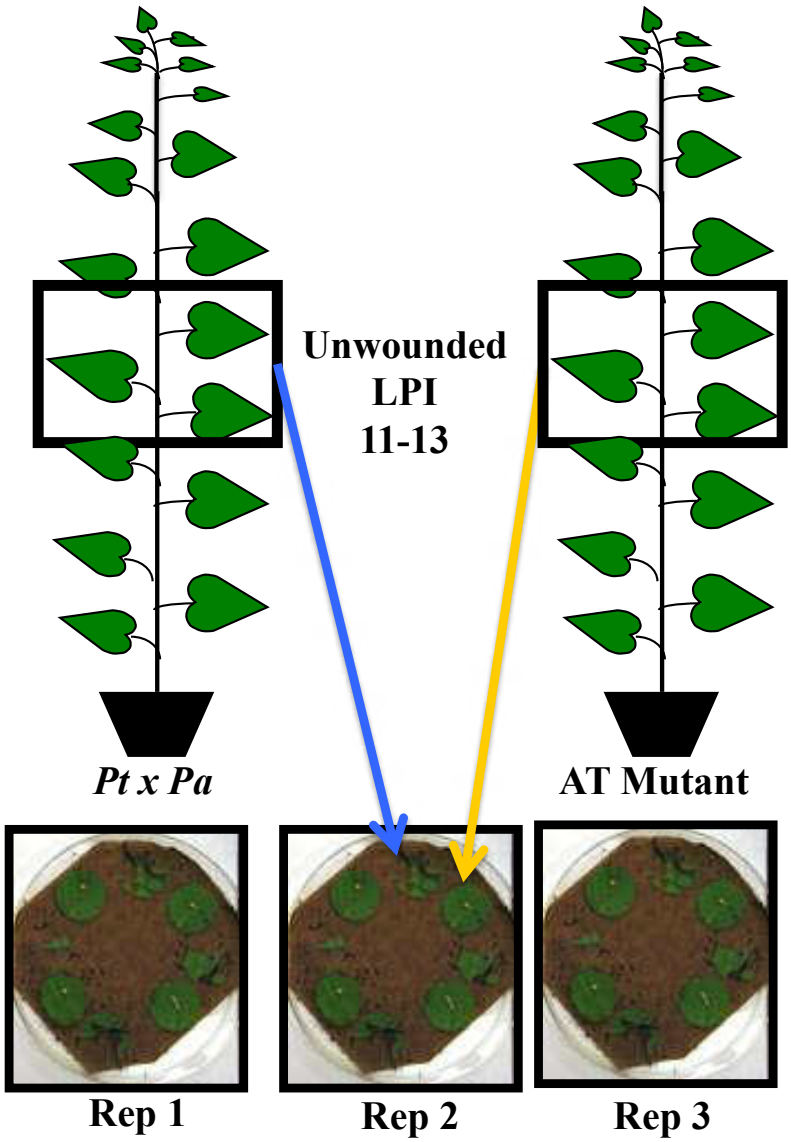

Figure 7: QU unwounded bioassay design. LPI 11-13 leaves from wildtype and mutant trees (paired biological replicates) were cut into leaf disks and arranged in an alternating pattern on petri plates (technical replicates). After 24 hours of starvation, five 3rd instar larvae were placed on each plate and allowed to feed until 30-50% of leaf material was consumed. Leaf weight consumed was calculated by weighing leaf disks and subtracting them from the average weight of unconsumed leaves. Leaf area consumed was calculated using GIMP imaging software to determine feeding preference.

and dried in an oven at 65°C for two days. These leaves were harvested to serve as a control for calculating percent leaf weight consumption after each choice assay. Five late 3rd to early 4th instar WMTM larvae were starved 24 hours prior to choice assays by

placing insects on petri plates with wet paper towels and then into a incubator (Percival Scientific model #166LVL; Perry, IA, USA) at 22°C, 50% humidity with a 12:12 light-dark cycle. Insects were allowed to feed for 24-48 hours until approximately 30-50% of the total leaf material was consumed. Mutant and *Pt x Pa* wildtype leaf material remaining from each petri plate were scanned and area remaining was determined using GIMP version 2.4.6 (www.gimp.org). Leaf material was then placed in paper bags and dried for two days at 65°C prior to weighing for the leaf weight consumption analysis. Nested ANOVA statistical tests for leaf weight and leaf area consumption were done using the R software program (www.r-project.org).

On-tree no-choice development bioassays. Two independent experiments of on-tree no-choice development bioassays were conducted to compare insect growth for WMTM reared on *E8-16* and *Pt x Pa* trees. In the first experiment, seven new copies of *Pt x Pa* and *E8-16* trees were propagated using air-layering and grown in the greenhouse to an approximate height of 100 cm. Nylon mesh bags were used to cage twelve large pre-weighed 2nd instar WMTM on LPI 8-16 for each tree. An additional twelve 2nd instar WMTM larvae were frozen and then dried in the oven at 65°C for two days for eventual dry weight gain measurements. Caged insects were allowed to feed until the largest insects were late fourth instar (11 days). Average wet weight gain of insects for each tree were measured before insects were frozen and then dried at 65°C for two days prior to measuring dry weight. A second independent experiment was conducted in the same manner with ten new biological copies per genotype in June 2011 over a seven day period.

Petri plate no-choice development bioassays. Two independent petri plate no-choice development bioassays, two weeks in length, were also completed to measure differences in larval development and leaf consumption of insects reared on *Pt x Pa* and *E8-16* leaf material. LPI 12 leaves for each genotype (10 trees per genotype for each experiment) were cut into 6.5 cm^2 leaf disks and placed on three technicial replicate petri plates per tree with moist paper towels. In the beginning of the experiment two leaf disks were placed on each replicate plate. As the experiment progressed the number of leaf disks placed on each plate increased to ensure leaf material was not limited. Eight WMTM, three days post hatch, were weighed, placed on each plate, and allowed to feed for 48 hours. Every two days leaf disks were scanned, dried, and weighed to measure leaf area and weight consumption. Leaf disks were replaced on plates from leaves located apically from the previous harvesting. WMTM instar stage and mortality was recorded every 48 hours and average wet weight measurements was taken on days 6, 10, and 14. After two weeks, insects were frozen and dried to measure dry weight gain.

MTU Screen

Plant propagation. Dr. Victor Busov generated a population of AT trees by transforming the AT vector pSKI074 into the *Pt x Pa* background using *Agrobacterium tumefasciens* (Busov et al. 2003). Unrooted stems were shipped to the University of North Dakota where stem cuttings were rooted and propagated in magenta boxes with media [2.15 g/L Mirashige-Skoog salts, 205 mg/L 2-(N-morpholino)ethanesulfonic acid, 100 mg/L myo-inositol, 10 mL FV vitamins (25 mg each of nictonic acid, pyridoxine HCL, thiamine HCL, L-cysteine, biotin, L-glutamine in 250 mL MilliQ water), 20 g/L sucrose, and 6.5 g/L Phytoblend agar] at pH 5.8 with 1 mL of Plant Preservative Mixture

antibiotics in an incubator (Percival Scientific model #CU36L5; Perry, IA, USA). Reagents for media were purchased from Caisson Laboratories, Smithfield, UT, USA. Incubator conditions were set for a 12:12 day-night light cycle with 40% humidity. Three copies of each mutant were propagated and then transferred to "4" pots with Sunshine Mix #1 soil. Trees were covered with bags and grown on racks under a 16:8 diurnal cycle using artificial light in the tissue culture room (figure 8). Trees were gradually acclimated to less humidity by removing bags slowly over a course of two weeks and then transferred to an environmental growth chamber for a few weeks (Percival Scientific; Perry, IA, USA). Trees were re-potted in one-gallon pots with a 3:1 ratio of Sunshine Mix #1 and sand, and then transferred to the Starcher Hall greenhouse. Each mutant triplet was randomly paired with another mutant triplet, arranged in an alternating pattern in a single row within the greenhouse, and grown together for eventual bioassays.

Regurgitant collection. Large 4th and 5th instar larvae were fed LPI 10-18 *Pt x Pa* wildtype leaves for 24 hours in petri plates in the growth chamber. Insects were then placed on a chilled glass petri dish and put into a 4ºC fridge for five to ten minutes to reduce their activity for easier handling. Cool temperatures also resulted in some insects expelling liquid onto the petri plate. Additional regurgitant was extracted from each insect by gently squeezing each insect behind the head to encourage regurgitation onto the plates. Each larvae produced approximately 20 microliters of regurgitant. Plates were then washed with 800 µL of dH2O, for a total of 1 mL, and then pipetted into 1.7 mL microcentrifuge tubes. One µL of Tween20 (VWR Internation; Radnor, PA, USA) was added to make the regurgitant easier to spread on leaves. Insect regurgitant was centrifuged at 10,000 rpm for five minutes at 4°C to remove insect hair, frass, or larger

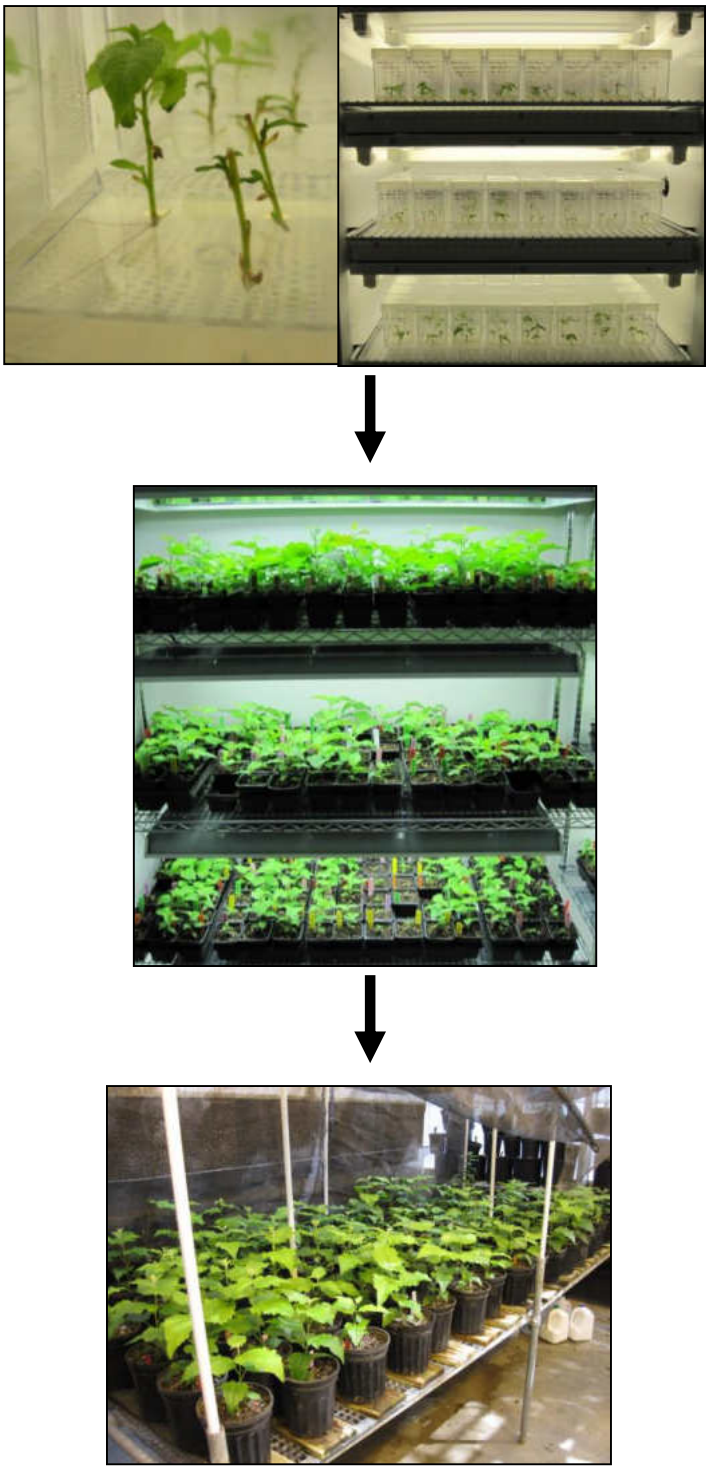

Figure 8: MTU plant propagation. Stems were placed in tissue culture until significant rooting. Rooted plantlets were put in "4" pots in the tissue culture room under artificial light. Mutants were eventually potted in 1 gallon pots and paired with another mutant triplet for eventual bioassays.

leaf matter and then pipetted into a clean microcentrifuge tube. Regurgitant was either immediately used or stored at -80ºC for future bioassays. Regurgitant was never reused once thawed.

Choice bioassay screen. After mutant pairs had grown 80-120 cm in height in the Starcher Hall greenhouse, three choice bioassays were conducted to identify mutants with altered consistutive and induced defenses (figure 9). Each choice bioassay consisted of three biological pairs with two technical replicate petri plates per pair. Unwounded choice bioassays were conducted as previously described using LPI 13-15 leaves. Five to six late 3rd to early 4th instar WMTM larvae were starved for 24 hours and then placed on each plate. Insects were allowed to feed until 40-60% of the total leaf material was consumed. Leaf area remaining for each mutant was scanned and analyzed using GIMP. The area remaining for one mutant was divided by the leaf area remaining for the second mutant in each pair to create a feeding choice ratio (CFR), as was done in Ralph (2009). Additional leaf disks were cut, dried in an oven, and served as control disks to measure leaf weight consumption. A leaf weight FCR was calculated by dividing the leaf weight remaining for one mutant over the second mutant.

Seventy-two hours later, three leaves located immediately above those harvested for the unwounded assay were wounded. A single wound line was made with a fabric pinwheel, approximately 10 cm in length, on either side of the midvein for each leaf. Ten microliters of insect regurgitant was then applied to each wound line (60 µL per tree) using a paintbrush. Twenty-four hours later wounded leaves were harvested from both mutants, cut into disks, and arranged on petri plates (wound local choice bioassay). At the same time LPI 7-9 leaves, located approximately 15-30 cm above the wounded leaves,

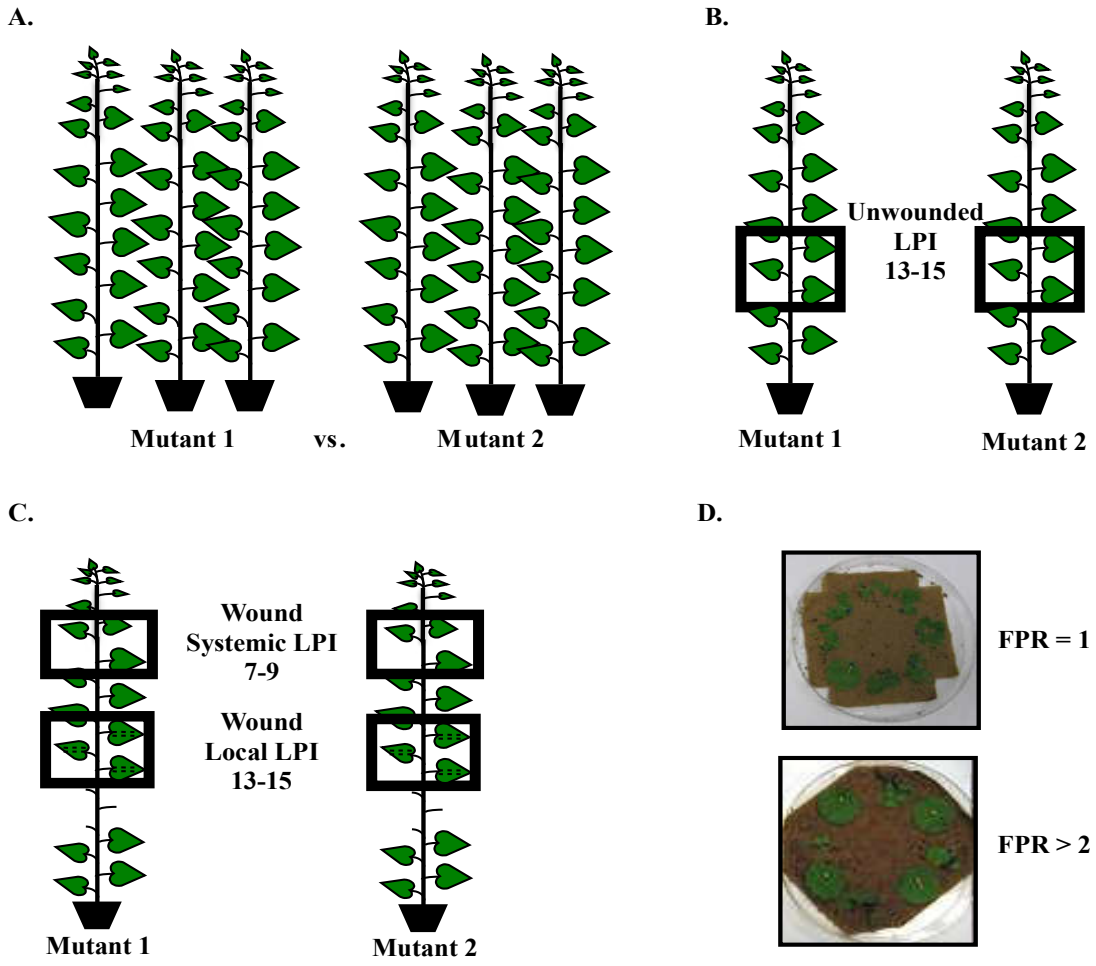

Figure 9: Unwounded, wound local, and wound systemic mutant-mutant bioassay screen design. A) Mutant-mutant pairs, three copies each, were randomly paired and grown together in the Starcher greenhouse. B) In the unwounded choice bioassay biological replicates for mutant 1 and mutant 2 were paired and LPI 13-15 was harvested and cut into disks with a copper pipe. Leaf disks from each mutant were placed in an alternating pattern on petri plates and secured with colored minuten pins. Five to six late third and early fourth instar WMTM larvae were placed onto each plate and allowed to feed until 40-60% of total leaf area on the plate was consumed. Leaf area remaining from mutant 1 was divided by leaf material remaining from mutant 2 to create a feeding choice ratio (FCR). C) Three days following the unwounded assay the new LPI 13-15 leaves (wound local choice bioassay) were wounded along both sides of the mid-vein and insect regurgitant was applied on each strip. Twenty-four hours later wounded local leaves were harvested and compared in wound local bioassays. LPI 7-9 were also harvested to test for systemic responses to wounding (wound systemic choice bioassay). Surface area and % weight FCRs were calculated similar to the unwounded response assay. D) Example of a plate showing no difference in WMTM feeding preference (FCR =1) and a plate with a strong preference (FCR > 2 std deviations).

were also harvested and used in a wound systemic choice bioassays. Choice bioassay set-up and analysis for wound local and wound systemic choice bioassay for leaf area were conducted in the same manner as the unwounded bioassays. Analysis for leaf weight consumption was slightly altered to take into account that the smaller sized leaves used in wound systemic bioassays and the physical wounding on portions of each leaf in wound local assays prevented cutting enough control disks. For wound local and wound systemic choice bioassays we used the control disks from the unwounded choice bioassay to determine amount of leaf weight consumed by WMTM. Leaf area and leaf weight FPRs for all mutant-mutant pairs were log transformed and binned by intervals of 0.05 for histograms for all three bioassays. Linear regressions comparing leaf area FCR and leaf weight FCR were performed using R. The mutant-mutant screen was conducted on 770 mutants between August 2010 and January 2013.

Validation of MTU mutants. Following the initial screen, mutant pairs that had a FPR $\geq$ 2 standard deviations from the population mean for a single assay, or $\geq$ 1.5 in multiple assays, were selected for choice bioassays against *Pt x Pa* wildtype trees (figure 10). The selection criteria was created to maximize the chance of finding mutants with a large effect on WMTM feeding preference in one bioassay, or a significant effect on feeding preference across multiple choice bioassays. Three new biological replicates of mutants, along with *Pt x Pa* wildtype trees, were propagated by air-layering and compared in unwounded, wound local, and wound systemic choice bioassays as previously described. Mutants that displayed greater than 40% difference in leaf area consumption compared to *Pt x Pa* wildtype trees and a nested ANOVA p value < 0.01 using R were selected for an additional round of choice bioassays with greater biological

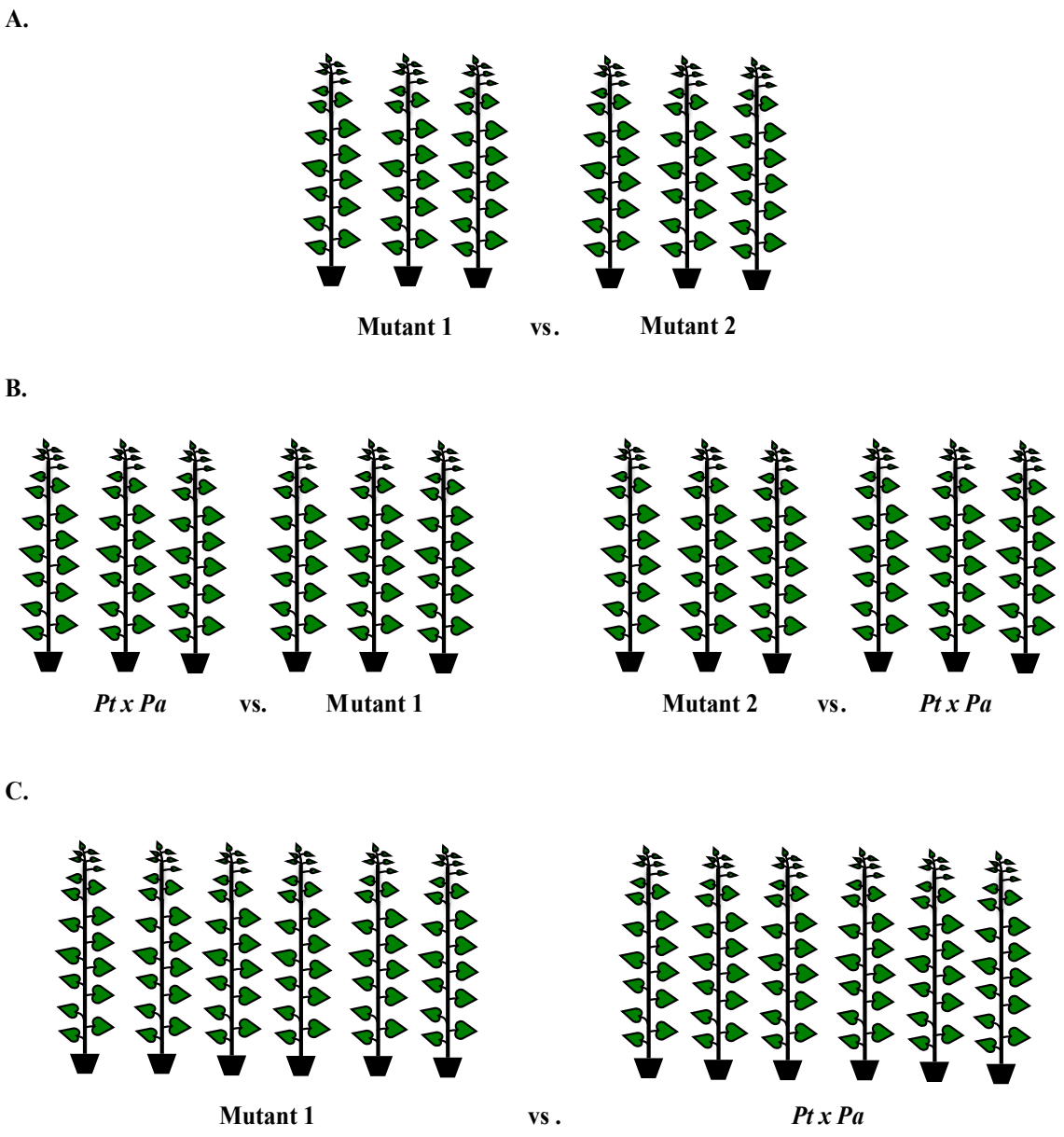

Figure 10: MTU screen and validation. A) Mutant triplets were paired and grown in the UND Starcher greenhouse and three choice bioassays (unwounded, wound local, and wound systemic) were performed for each mutant-mutant pair. B) If WMTM had a preference to consume leaf material from one mutant over the other mutant in any of the three bioassays then new copies for both mutants were propagated and compared against *Pt x Pa* wildtype trees in choice bioassays. C) Mutants displaying greater than 40% difference in leaf area consumption compared to *Pt x Pa* wildtype trees and a nested ANOVA p value < 0.01 were selected as candidates for an additional round of choice bioassays with greater biological replication.

replication. Four candidates, *237p-15*, *239L-5*, *173L-4*, and *357L-1* had unwounded, wound local, and wound systemic choice assays performed with greater biological replication (6-10 biological replicates per tree) as previously described.

An on-tree no-choice development bioassay was also conducted on *357L-1* to determine if the mutant had an impact on insect development and weight gain. Twelve 2nd instar WMTM larvae were caged with mesh bags on LPI 8-16 leaves of seven *357L-1* and *Pt x Pa* wildtype trees. Insects were caged on trees for eight days until the largest insects were late 4th instar. Instar stage was recorded for each insect and wet weight gain was calculated as previously described. A t-test comparing weight gain between insects caged on each genotype was calculated using R.

Results and Discussion

QU Candidates

Unwounded choice bioassays. Nine AT mutants identified from the screen at QU (*E14-56*, *E7-4*, *E8-13*, *E8-16*, *EB25-9*, *R22-6*, *R1-1*, *B7-17*, and *R8-23*) were tested using unwounded choice bioassays. One independent experiment was performed for each candidate to determine if WMTM had a preference for *Pt x Pa* over the mutant for both leaf area and leaf weigh consumption. A second independent unwounded choice bioassay was only performed if there was a substantial feeding preference difference (≥ 30%) between the mutant and *Pt x Pa* with a p value < 0.05 for both the leaf area and leaf weight consumption data in the first experiment. Eight mutants did not have significant feeding difference or significant statistical support to continue with additional bioassays (table 2). WMTM did show more preference for *Pt x Pa* leaves than *E8-16* in two independent unwounded choice bioassays for both leaf area and leaf weight (figure 11A).

Table 2: WMTM did not show a feeding preference in unwounded choice bioasays for eight QU candidates. Candidates (*E7-4*, *E8-13*, *EB25-9*, *R22-6*, *R1-1*, *B7-17*, *R8-23*, *E14-56*) selected from the screen at QU, propagated with *Pt x Pa* wildtype trees, and were tested in independent choice bioassays to determine if WMTM had a preference between the mutant candidate and paired wildtype trees. Choice assays were designed to These mutants did not meet our criteria for having a substantial feeding preference difference (≥ 30%) between the mutant and *Pt x Pa* with a p value < 0.05 for both the leaf area and leaf weight consumption data.

Mutant	Leaf Area	Leaf Weight
E7-4	Not significant, p=0.08, n=6 pairs	Not significant, p=0.69, n=6 pairs
E8-13	Not significant, p=0.28, n=6 pairs	Not significant, p=0.56, n=6 pairs
EB25-9	Not significant, p=0.12, n=9 pairs	Not significant, p=0.20, n=9 pairs
R22-6	Significant, p=0.002, n=6 pairs, WMTM fed 23.8% more on mutant	Significant, p=0.018, n=6 pairs, WMTM fed 22.4% on mutant
R1-1	Not significant, p=0.1, n=6 pairs	Significant, p=0.031, n=6 pairs, WMTM fed 22% more on mutant
B7-17	Not significant, p=0.07, n=6 pairs	Not significant, p=0.31, n=6 pairs
R8-23	Significant, p=0.001, n=7 pairs, WMTM fed 21.7% more on mutant	Not significant, p=0.4, n=7 pairs
E14-56	Not significant, p=0.29, n=7 pairs	Not significant, p=0.25, n=7 pairs

In the first round of choice assays we observed 35% less WMTM feeding in leaf area (Nested ANOVA, p=0.0038, n=6 pairs) and 34% less feeding in leaf weight (Nested ANOVA, p=0.003, n=6 pairs) from *E8-16* than *Pt x Pa*. WMTM consumed 68.8% more

leaf weight from *Pt x Pa* leaf disks than *E8-16* (figure 11A; Nested ANOVA, p<0.001). In the second round we observed 44% less feeding in leaf area (figure 11B; Nested ANOVA, p<0.001, n=6 pairs) and 68% less feeding in leaf weight (figure 11B; Nested ANOVA, p<0.001, df=6 pairs) on *E8-16* trees. Because insects did not prefer to feed on

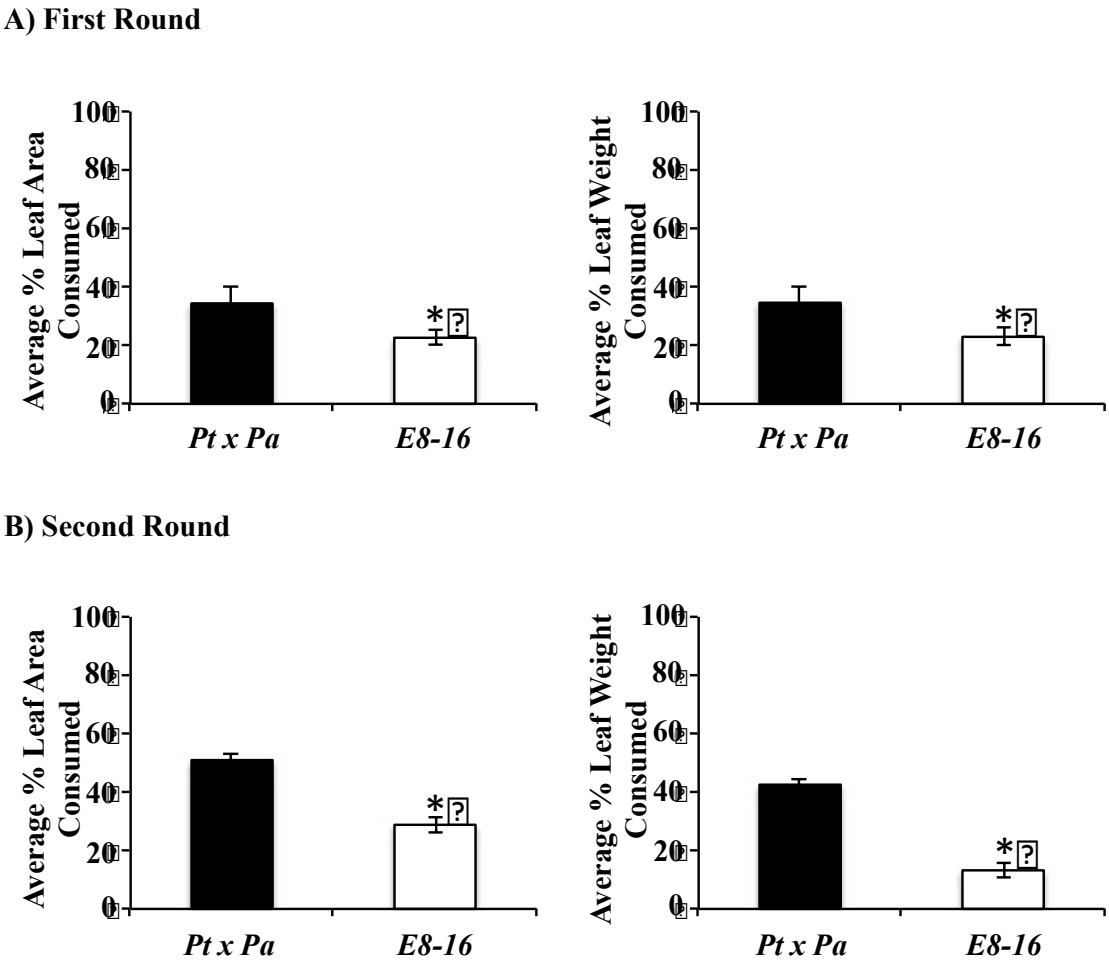

Figure 11: WMTM preferred to consume unwounded leaves from *Pt x Pa* wild-type trees over *E8-16* in choice bioassays. Two independent of unwounded choice bioassays were conducted to determine if WMTM larvae had a preference between the mutant and *Pt x Pa*. A) In the first round of choice assays we observed 35% less feeding in leaf area (Nested ANOVA, p=0.0038, n=6 pairs) and 34% less feeding in leaf weight (Nested ANOVA, p=0.003, n=6 pairs) on *E8-16* trees than *Pt x Pa*. B) In the second round we observed 44% less feeding in leaf area (Nested ANOVA, p<0.001, n=6 pairs) and 68% less feeding in leaf weight (Nested ANOVA, p<0.001, df=6 pairs) on *E8-16* trees. Bars represent mean +/- SE in panels A and B. * indicate statistical significance

E8-16 leaves we continued with on-tree and petri plate no-choice development bioassays to determine if *E8-16* had a negative impact on WMTM growth and development.

On-tree and petri plate no-choice development bioassays. In the first on-tree no-choice development bioassay we compared growth of WMTM larvae when caged on either *Pt x Pa* wildtype trees or *E8-16* trees, over 11 days. WMTM did not gain more wet weight (figure 12A; Two sample t-test, $p>0.05$) or dry weight (figure 12A; Two sample t-test, $p>0.05$) per insect on *Pt x Pa* wildtype trees than *E8-16*. In the second round of on-tree no-choice bioassays we increased the number of biological replicates to ten *E8-16* and *Pt x Pa* wildtype trees. WMTM larvae caged on *Pt x Pa* trees gained 40.7% more wet weight per insect (figure 12B; Two sample t-test, $p=0.0002$, $n=10$ trees each) and 43.6% more dry weight than insects reared on *E8-16* trees (figure 12B; Two sample t-test, $p=0.0003$, $n=10$ trees each).

In the petri plate no choice development bioassay *E8-16* had a negative impact on insect weight gain and development. WMTM larvae consumed more *Pt x Pa* leaf weight on days 4, 8, 10, 12, and 14 than *E8-16* (figure 13A; Nested ANOVA $p<0.01$, n= *E8-16* and *Pt x Pa* trees) and consumed more leaf area from *Pt x Pa* than *E8-16* trees on days 10, 12, and 14 (figure 13B; Nested ANOVA $p<0.001$). Wet weight of WMTM larvae was measured on days 6, 10, and 14 of the two week bioassay. WMTM gained 34.6% less wet weight on *E8-16* wildtype leaves on day 6 (figure 13C; Nested ANOVA, $p<0.001$) than larvae reared on *Pt x Pa* wildtype leaves. WMTM wet weight gain was also 44.8% less on day 10 ($p<0.00001$), and 44.4% less on day 14 ($p<0.00001$) on *E8-16* leaves compared to *Pt x Pa* wildtype. After the two week bioassay WMTM were dried and weighed to determine dry weight gain. WMTM gained 53% less dry weight gain on *E8-*

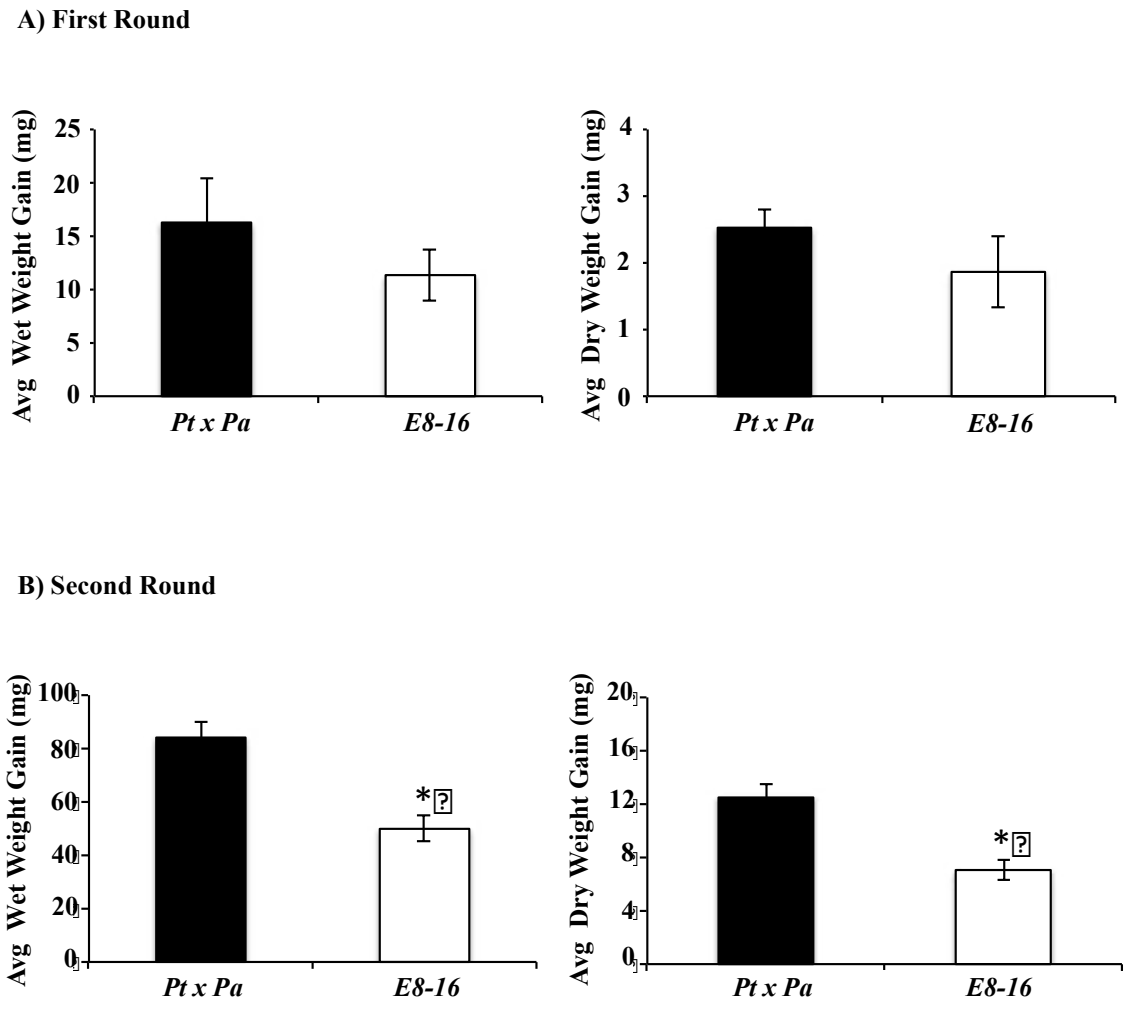

Figure 12: Results for two independent on-tree no-choice bioassays comparing WMTM weight gain and development when reared on either *E8-16* or *Pt x Pa* trees. A) In the first experiment of on-tree assays there was a small biological difference but not a statistical difference in average larval wet weight gain (two sample t-test, p=0.3206, df=12) and average larval dry weight gain (Welch t-test, p=0.3433, df=12). B) When we conducted a second experiment we included more biological replication. Over a 7 day period we observed WMTM larvae gain 40.7% more wet weight per insect on *Pt* x *Pa* trees (Two sample t-test, p=0.0002) and 43.6% more dry weight than insects reared on *E8-16* trees (Two sample t-test, p=0.0003). Bars represent mean +/- SE in panels A and B. * indicate statistical significance

16 leaves than *Pt x Pa* (figure 13D; Nested ANOVA, p<0.001). WMTM larvae also developed faster on *Pt x Pa* leaves than *E8-16* leaves by day 14 (figure 15E, Chi Square Test, p<0.0001).

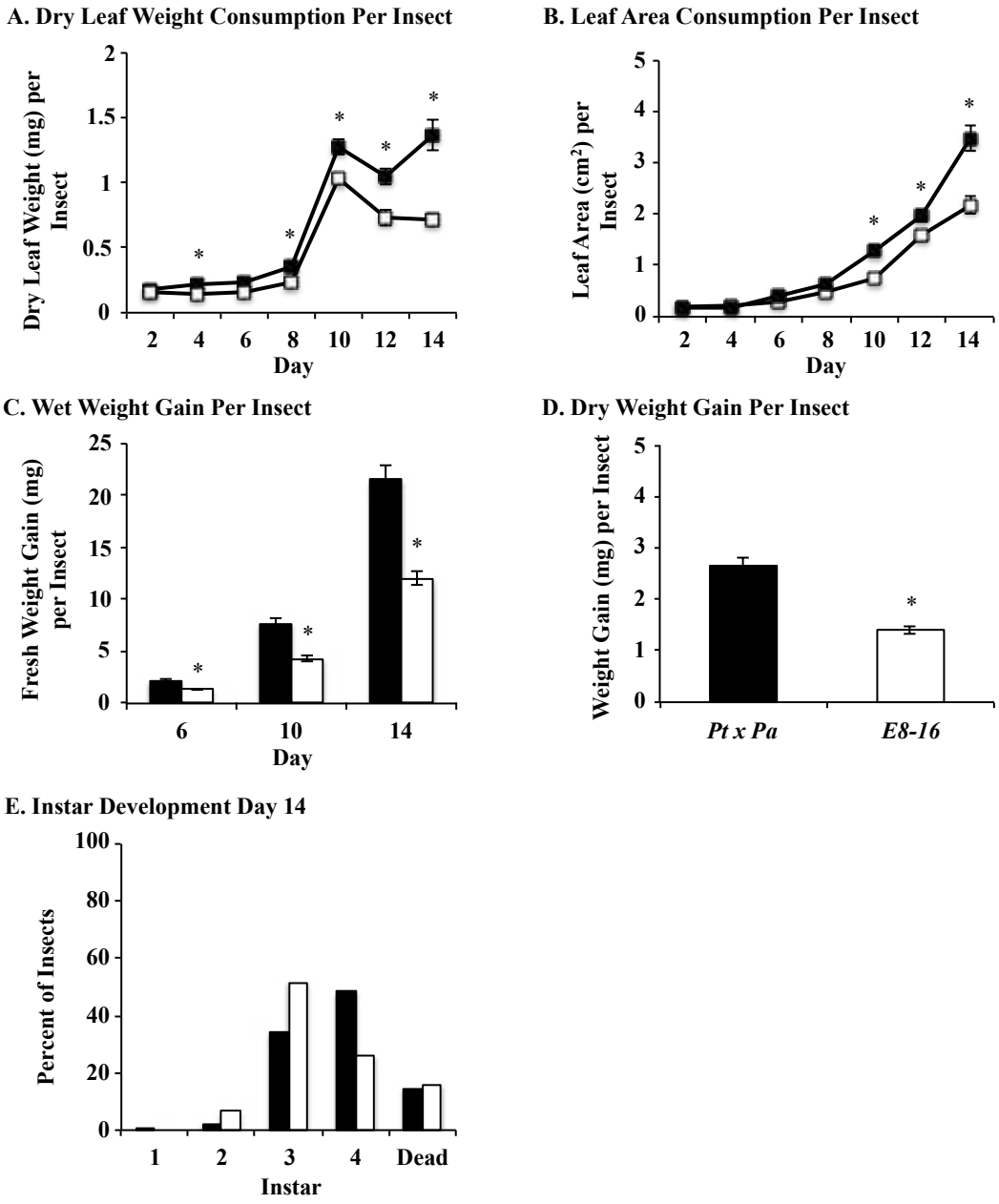

Figure 13: WMTM larvae prefer feeding, consume more, and develop faster on *Pt x Pa* wild-type leaves than *E8-16* leaves in the first petri plate no-choice development bioassay. A) WMTM larvae consumed more *Pt x Pa* leaf weight on days 4, 8, 10, 12, and 14 than *E8-16* (Nested ANOVA $p<0.01$, n= 10 *E8-16* and *Pt x Pa* trees). B) WMTM consumed more leaf area from *Pt x Pa* than *E8-16* trees on days 10, 12, and 14 (Nested ANOVA $p<0.05$). C) WMTM gained more wet weight on *Pt x Pa* leaves on days 6, 10, and 14 than insects raised on *E8-16* leaves (Nested ANOVA, $p<0.001$). D) WMTM gained 53% less dry weight gain on *E8-16* leaves than *Pt x Pa* (Nested ANOVA, $p<0.001$). E) WMTM larvae developed faster on *Pt x Pa* leaves than *E8-16* leaves by day 14 (Chi Square Test, $p<0.0001$). Black bars and squares indicates *E8-16*, white indicates *Pt x Pa*. Bars represent mean +/- SE in panels A and B. * indicate statistical significance.

Results for the second petri plate no-choice development bioassay were similar to first experiment. WMTM larvae consumed more *Pt x Pa* leaf weight on days 6, 10, and 12 than *E8-16* (figure 14A; Nested ANOVA $p<0.005$, n=10 *E8-16* and *Pt x Pa* trees) and more leaf area from *Pt x Pa* than *E8-16* trees on days 10 and 12 (figure 14B; Nested ANOVA $p<0.05$). WMTM gained 27.9% less wet weight on day 6 (figure 14C; Nested ANOVA, $p<0.01$), 28.7% less on day 10 (Nested ANOVA, $p<0.001$), and 25.5% less on day14 (Nested ANOVA, $p<0.001$) on *E8-16* leaves than *Pt x Pa* wildtype leaves. WMTM also gained 30% less dry weight on *E8-16* leaves than *Pt x Pa* (figure 14D; Nested ANOVA, $p<0.01$). WMTM larvae also developed faster on *Pt x Pa* leaves than *E8-16* leaves by day 14 (figure 14E; Chi Square Test, $p<0.001$).

Bioassay results suggest that WMTM preferred to consume leaves from *Pt x Pa* wild-type trees over *E8-16*. Less leaf consumption of *E8-16* appears to result in a negative impact on larval weight gain and development. Results from bioassays are relatively consistent suggesting that this is a prime candidate for molecular characterization to identify T-DNA location and the "activated" gene.

Michigan Tech Screen

Leaf area vs. leaf weight FCR comparison. In the beginning of our screen we examined whether leaf area could be used as an alternative to leaf weight to identify AT genotypes with altered WMTM feeding preference. Measuring leaf weight consumption, while more biologically relevant, has drawbacks including: 1) time required to dry and weigh leaf disks, 2) accuracy in manually weighing small leaf fragments that can crush easily or get stuck within bags, and 3) the impracticality of long term storage of leaf disks to confirm significant results. Measuring leaf area consumption by scanning leaf disks

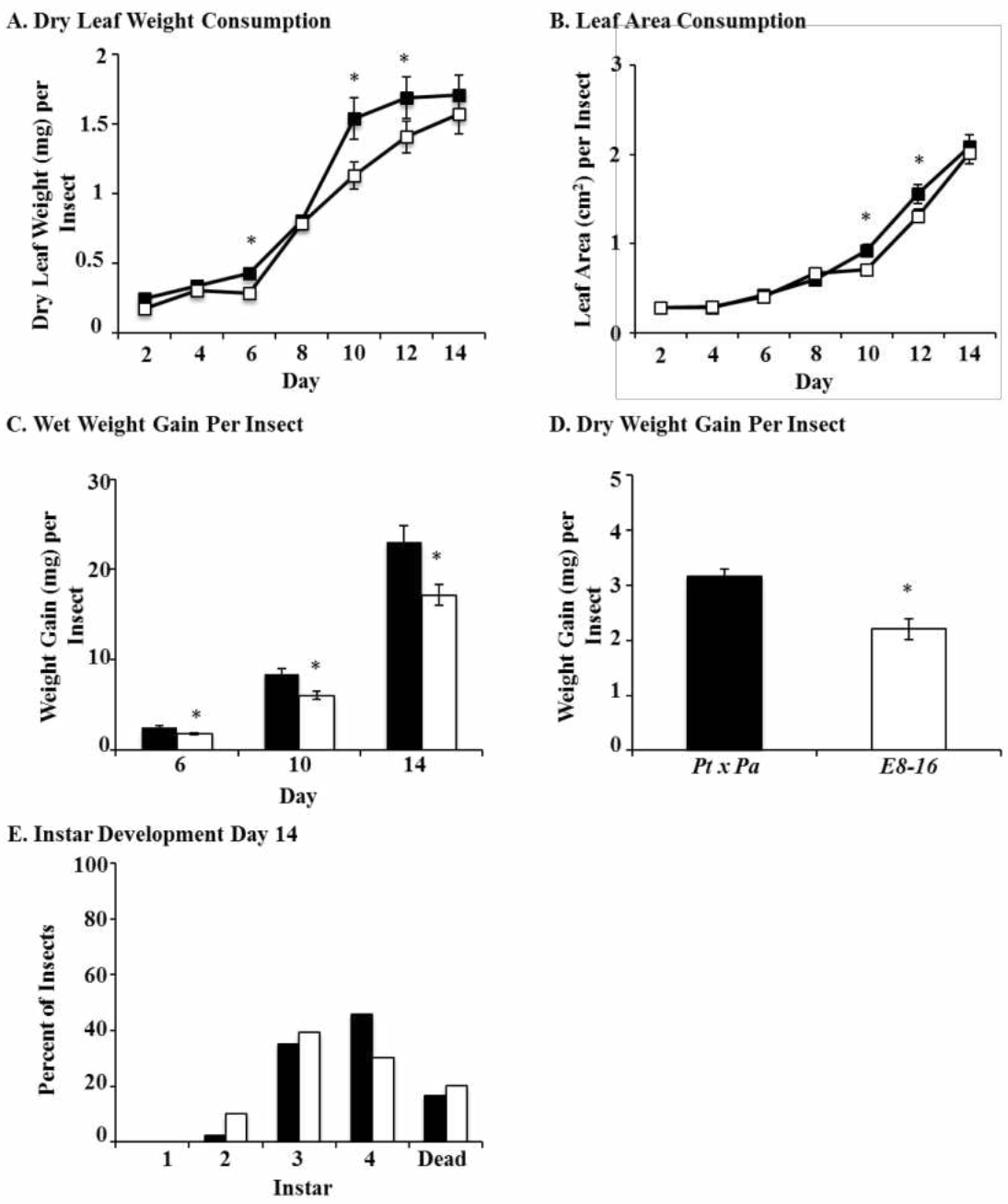

Figure 14: WMTM larvae prefer feeding, consume more, and develop faster on *Pt x Pa* wild-type leaves than *E8-16* leaves in the second independent petri plate no-choice development bioassay. A) WMTM larvae consumed more *Pt x Pa* leaf weight on days 6, 10, and 12 than *E8-16* (Nested ANOVA $p<0.05$, n=10 *E8-16* and *Pt x Pa* trees). B) WMTM consumed more leaf area from *Pt x Pa* than *E8-16* trees on days 10 and 12 (Nested ANOVA $p<0.05$). C) WMTM gained more wet weight on *Pt x Pa* leaves on days 6, 10, and 14 than insects raised on *E8-16* leaves (Nested ANOVA, $p<0.05$). D) WMTM gained 30% less dry weight gain on *E8-16* leaves than *Pt x Pa* (Nested ANOVA, $p<0.01$). E) WMTM larvae developed faster on *Pt x Pa* leaves than *E8-16* leaves by day 14 (Chi Square Test, $p<0.001$). Black bars and squares indicates *E8-16*, white indicates *Pt x Pa*. Bars represent mean +/- SE in panels A and B. * indicate statistical significance.

allows for faster processing of data, minimal manual handling of leaf disks and calculations, and data can be stored digitally for future analysis. For the first 43 mutant-mutant comparisons tested we determined FCRs using leaf area and leaf weight for each bioassay and compared them using Pearson correlation tests (figure 15). We identified a significant relationship between area and weight FCRs for unwounded (Pearson Correlation $F_{1,42}$=337.73, p=0.00, R^2=0.891, y=0.92745x-0.00293), wound local (Pearson Correlation $F_{1,42}$=182.07, p=1.11E-16, R^2=0.816, y=0.92x+0.00199), and wound systemic (Pearson Correlation $F_{1,42}$=174.64, p=2.22E-16, R^2=0.81, y=0.971x+0.00336) assays (figure 15). A strong correlation between area and weight FCR, despite using control disks from unwounded assays for all FCR calculations, suggested that we could continue with the screen by only analyzing leaf area FCR comparisons and not miss any true positives.

Mutant-mutant screen. The purpose of conducting any large-scale screening effort is to rapidly identify candidates for further biological and molecular characterization while limiting the number of false positives. In an ideal situation each AT mutant would be compared against wildtype trees with enough biological and technical replication to identify true positives in a single round of bioassays. However, this would be impractical and inefficient for reasons of time, money, and greenhouse space. Instead, mutant-mutant comparisons were conducted with the assumption that a significant majority of mutants would not impact WMTM feeding preference (Ralph 2009). If we were to expect as much as 5% of mutants to have altered IR, then the odds of two IR mutants being randomly paired together would be 0.05 x 0.05, or 0.25%. Therefore, out of a screen of 400 mutant pairings, only one pairing on average would

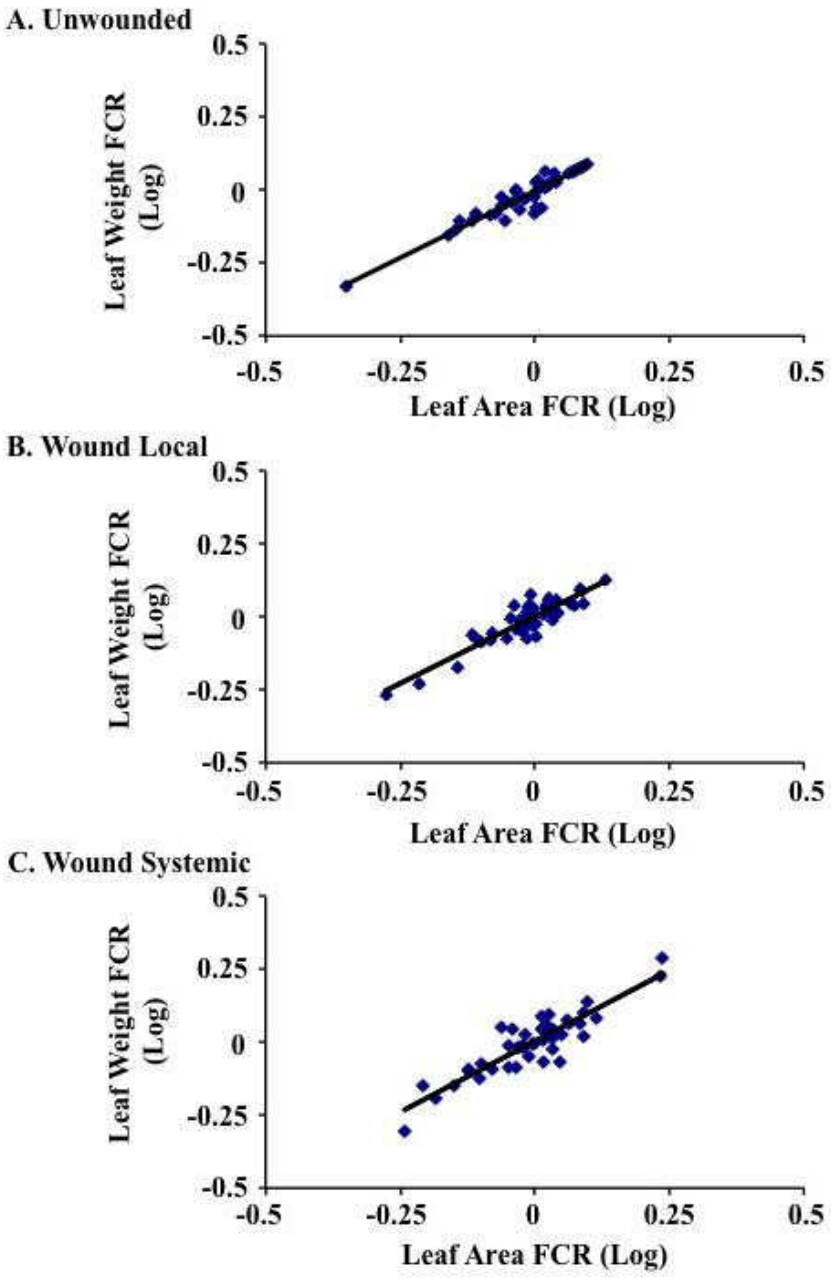

Figure 15: Comparison of FCR for 43 mutant pairs using leaf area and leaf weight data for unwounded, wound local, and wound systemic assays. Leaf area FPR is strongly correlated with leaf weight FCR for A) unwounded ($F_{1,42}$=337.73, p=0.00, R^2=0.891, y=0.92745x-0.00293), B) wound local ($F_{1,42}$=182.07, p=1.11E-16, R^2=0.816, y=0.92x+0.00199), and C) wound systemic bioassays ($F_{1,42}$=174.64, p=2.22E-16, R^2=0.81, y=0.971x+0.00336) despite using unwounded control leaf disks for each assay.

consist of two IR mutants. This would suggest that the risk of missing canididates with altered IR due to being paired together was remote. The odds of pairing an IR mutant with a non-IR mutant would be 9.5% (0.05 x 0.95 + 0.95 x 0.05). Out of 400 pairings that would equal 38 pairs. By comparison, if we were to compare wildtype trees against each mutant in the AT population, then this would result in 20 mutant pairs (5%). Therefore mutant-mutant pairings would be sufficient to find mutants with altered IR. Three separate choice bioassays (unwounded, wound local, and wound systemic) were used to identify mutants with altered IR.

Histograms showing the distribution of FCRs for 770 mutants (385 pairs) revealed a normal distribution curve for each class of choice bioassay (figure 16A-C) with the majority of mutant pairs at or near a FCR of 0. In unwounded choice bioassays the mean FPR was 0 ± 0.09 with forty-four mutants (22 pairs, 5.7%) having a FCR ≥ two standard deviations from the mean (figure 16A). Forty-four mutants (22 pairs, 5.7%) were identified as having a FCR greater than two standard deviations from the mean (0 ± 0.09, figure 16B) in wound local choice bioassays and forty-eight mutants (24 pairs, 6.2%) in wound systemic choice bioassays (mean= 0.01 ± 0.1, figure 16C). Mutants with a FCR ≥ 2 standard deviations from the mean for a single bioassay or ≥ 1.5 for multiple bioassays were selected as candidates for further investigation using bioassays against *Pt x Pa* wildtype trees. Between all three choice bioassays we identified 108 mutants (54 pairs) candidates (figure 16D). Fifty-four candidates (50%) had a FCR ≥ 2 standard deviations from the mean for a single bioassay while the remaining candidates had a FCR ≥ 1.5 for two (31.5%) or all three (18.5%) choice bioassays.

In total, our mutant-mutant screen identified 14% of the total AT population as

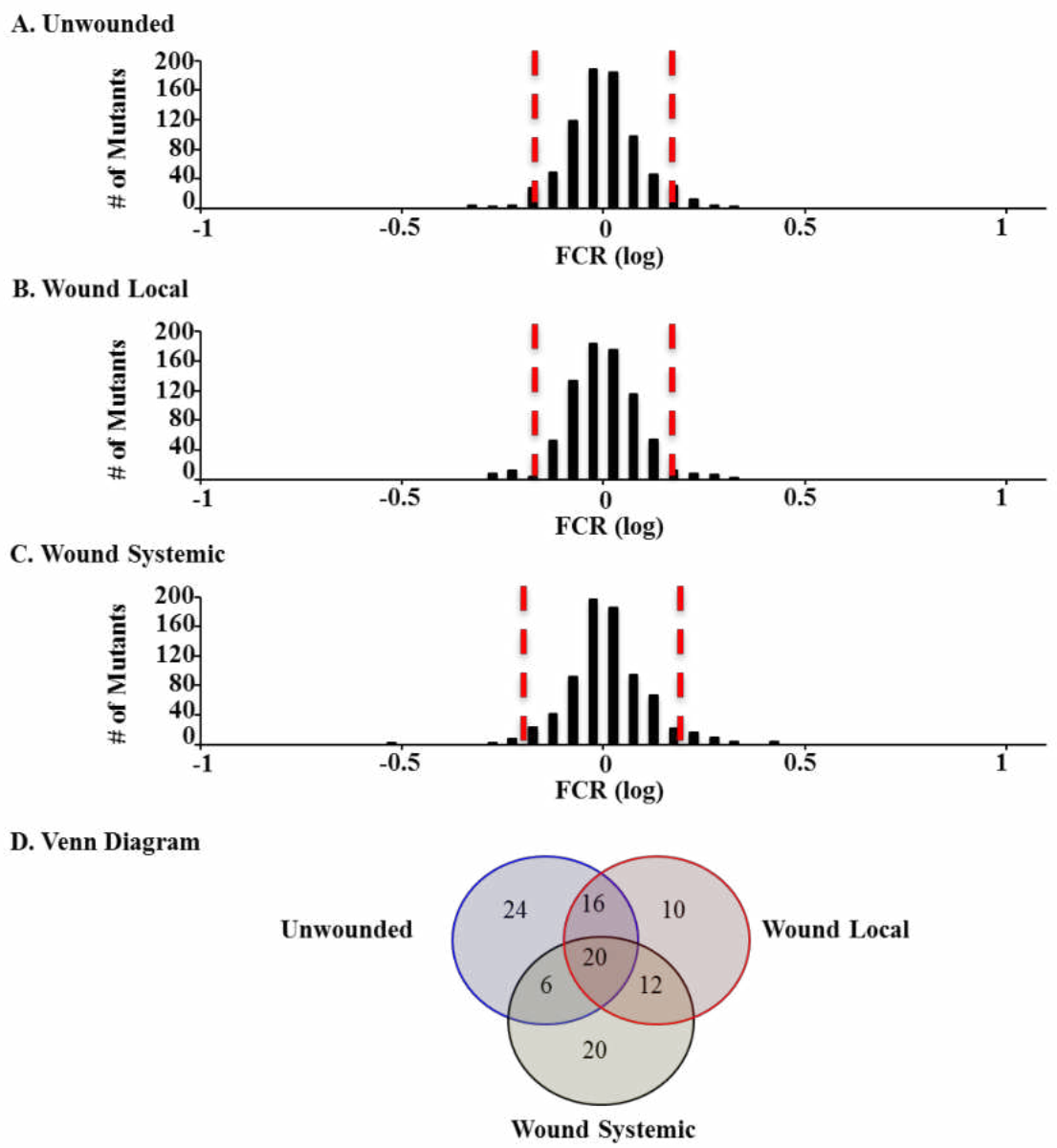

Figure 16: Mutant-mutant FCR histograms from unwounded, wound local, and wound systemic choice assays from the MTU AT population. A) In total 770 mutants (385 pairs) underwent choice assays. FPR data for all bioassays were log transformed and binned by units of 0.05 for histograms. In unwounded assays the mean FCR was 0 ± 0.09. The FCR values of 44 mutants (22 pairs) were outside two standard deviations and were identified as mutants for comparison against *Pt x Pa* wild-type trees. B) In wound local assays (mean 0 +/- 0.09) the FCR of 44 mutants (22 pairs) were greater than two standard deviations from the mean. C) The FCR values for 48 mutants (24 pairs) were outside two standard deviations from the population mean in wound systemic choice bioassays (mean= 0.01 +/- 0.1). Dashed red lines in panels A-C indicate two standard deviations from the mean. D) Venn diagram showing the 108 mutants that passed the FCR threshold (> 2 std deviations for one bioassay or >1.5 std deviations for two or more assays) in the initial mutant-mutant screen organized by bioassay type.

candidates for further validation. We had predicted that 9.5% of the 385 mutant-mutant pairings (36 pairs) would be IR-non-IR pairings. The 54 pairs we identified in the screen is higher than the predicted number of IR-non-IR mutant pairings we would have expected from the AT population. This could indicate that there are a number of false positives within our candidates. Half of the candidates had a signficant FCR for a single choice bioassay, possibly indicating a very specific role in constitutive or wound-induced defenses. The other half of candidates revealed a significant FCR for two or more bioassays with most of those being involved in local and systemic defense. Several microarray studies examining transcriptomes of *Populus* after mechanical wounding or insect feeding have shown many different types of genes involved in inducible defenses including kunitz protease inhibitors, endochitinases, leucine-rich repeats, ABC proteins,PPO, P450s, and enzymes involved in octadecanoid biosynthesis and phenylpropanoid metabolism (Major and Constabel 2006, Ralph et al. 2006, Babst et al. 2009). Within these gene families, such as the kunitz protease inhibitors and endochitinases, are members with complex function in regards to plant defense, that are present consitutively, can be induced at the site of feeding, and can be induced systematically following feeding (Phillipe et al. 2009, Christopher et al. 2004). With a more manageable population of interesting candidates we could now validate mutants within each pair against wild-type trees and with increased biological replication to confirm previous screening results and eliminate false positives.

Validation of candidates. Bioassays are inheritably variable. Health of trees and insects, as well as insect behavior are difficult to control. These variables could result in false positives amongst our candidates. In addition, our initial screen only identified

mutant pairs where WMTM preferred to consume leaves from one mutant over the other. The most likely scenario for any of these mutant-mutant pairs is that one mutant is resistant or sensitive to WMTM feeding and is paired with a mutant that is phenotypically wildtype with regards to IR. However, without additional validation comparing each mutant against a common background, we cannot know which mutant in each pair has altered IR. Therefore, independent bioassays were conducted by comparing mutants identified from the screen against the *Pt x Pa* wildtype background.

Twenty-one of the original 108 mutants identified from the initial screen were compared against *Pt x Pa* wild-type trees in unwounded, wound local, and wound systemic choice bioassays (table 3). These twenty-one mutants were selected as they were identified early in the screen to be a part of mutant-mutant pairs with significant FPR values. Three new biological replicates for each candidate were propagated along with *Pt x Pa* wildtype trees using the air-layering method and grown in the greenhouse. In this round of validation, mutants that had a $\geq 40\%$ difference in feeding preference compared to *Pt x Pa* wildtype, along with a nested ANOVA p-value <0.01 for at least one choice bioassay, were selected as candidates for additional bioassays with greater biological replication. Selection criteria were created to find the most interesting mutants among the 21 tested. Eight (*173L-4*, *237p-15*, *239L-4*, *357L-1*, *23L-3*, *372L-1*, *612L-1*, *175st-5*) of the 21 mutants (38%) were verified as candidates for future greenhouse and molecular work, with seven showing sensitivity in one assay (Table 3). Five of the eight candidates showed a statistically significant difference in WMTM feeding preference compared to *Pt x Pa* wildtype in wound systemic choice bioassays. One candidate was found in wound local bioassays, and two in unwounded bioassays. Mutants that did not

Table 3: Candidates identified from the MTU screen. Twenty-one candidates identified from the initial mutant screen were tested against *Pt x Pa* in unwounded, wound local (w. local) and wound systemic (w. systemic) choice bioassays. Three biological replicates were propagated for mutant lines and paired with *Pt x Pa* for each candidate. Mutants that had a ≥ 40% difference in feeding preference (% diff) and a p value < 0.01 for at least one bioassay were selected as candidates for additional bioassays with greater biological replication. * indicates mutants that passed our selection criteria.

	Unwounded		**W. Local**		**W. Systemic**	
Mutant	**p value**	**% Diff**	**p value**	**% Diff**	**p value**	**% Diff**
*173L-4**	< 0.05	-37.6	< 0.01	-42.8	> 0.05	-16.9
93L-1	< 0.05	-39.4	> 0.05	-19.2	> 0.05	6.8
*237p-15**	< 0.001	65.5	< 0.05	49.6	> 0.05	22.9
*239L-4**	< 0.05	33.3	< 0.05	65.6	< 0.005	169.9
238L-4	> 0.05	11.7	> 0.05	13.5	> 0.05	16.1
213L-1	> 0.05	-10.5	> 0.05	-15.9	> 0.05	-29.4
542L-3	< 0.05	57.1	> 0.05	-5.8	> 0.05	27.3
300L-5	> 0.05	0.7	> 0.05	7.6	> 0.05	19.1
240L-6	> 0.05	26.5	> 0.05	13.4	> 0.05	-9.6
176st-3	> 0.05	1.9	> 0.05	25.2	> 0.05	-10.3
171L-3	> 0.05	-8.6	> 0.05	27.8	> 0.05	38.8
377L-2	> 0.05	16.1	> 0.05	10.7	> 0.05	19.3
*357L-1**	> 0.05	76.2	< 0.05	105.2	< 0.001	150.0
627L-1	< 0.005	-34.0	> 0.05	-13.9	> 0.05	-9.7
*23L-3**	> 0.05	49.8	> 0.05	26.8	< 0.005	245.2
317L-4	> 0.05	73.8	< 0.05	60.2	> 0.05	60.5
*372L-1**	< 0.01	-55.5	> 0.05	-8.5	> 0.05	39.2
372L-2	< 0.01	-30.1	> 0.05	-27.6	> 0.05	-23.6
*612L-1**	> 0.05	34.4	> 0.05	40.7	< 0.00005	172.2
525L-3	> 0.05	18.8	> 0.05	32.4	> 0.05	-6.9
*175st-5**	> 0.05	-11.9	> 0.05	26.0	< 0.0001	80.2

meet our criteria were considered false positives. Some mutants did have either a ≥ 40% difference in WMTM feeding preference or a p value less than 0.001 for one of the three bioassays. These mutants may be of interest if followed up with additional bioassays with greater biological replication. However, due to time and financial constraints, we opted to focus on mutants that met our criteria having a large, statistically significant difference in insect feeding preference. Interestingly, WMTM preferred to feed on six of the mutants

(*237p-15*, *239L-4*, *357L-1*, *23L-3*, *612L-1*, *175st-5*) over *Pt x Pa*. That is, they appear to be sensitive to insect feeding. As shown in a study by Busov et al. (2010), approximately 30% of AT T-DNA insertions were within the promoter and coding regions of genes. Perhaps several of these mutants are not actually over-expressing a gene but are actually losing a copy of that particular gene. Perhaps insect sensitivity could result from the knock-down of a defense gene or the activation of a gene for growth/photosynthesis that alters the allocation of energy resources to plant defense. Further work to validate these mutants through bioassays and molecular characterization will likely reveal a diversity of genes that influence *Populus* defense.

Four of the eight candidates have been propagated to 6-10 biological copies and further tested against *Pt* x *Pa* trees using choice bioassays. *237p-15* was found to be sensitive in unwounded and wound local bioassays when compared against *Pt x Pa* wildtype with three biological replicates but did not show difference in bioassays with greater biological replication. In unwounded, wound local, and wound systemic choice bioassays using seven biological replicates WMTM larvae showed no consumption preference between *237p-15* and *Pt x Pa* wildtype leaves (figure 17; Nested ANOVA, $p>0.05$, n=6 pairs). In the original mutant-wildtype validation (three biological replicates) *239L-4* showed insect sensitivity in all three bioassays. In the first round of choice bioassays with greater biological replication WMTM showed no preference for *239L-4* leaves over *Pt x Pa* wildtype leaves in unwounded and wound systemic choice bioassays (figure 18; Nested ANOVA, $p>0.05$, n=6 pairs). WMTM did consume more 30% more leaf area from *239L-4* trees than *Pt x Pa* wildtype (Nested ANOVA, $p<0.005$, n=7 pairs). However, in a second independent set of choice bioassays we observed different results.

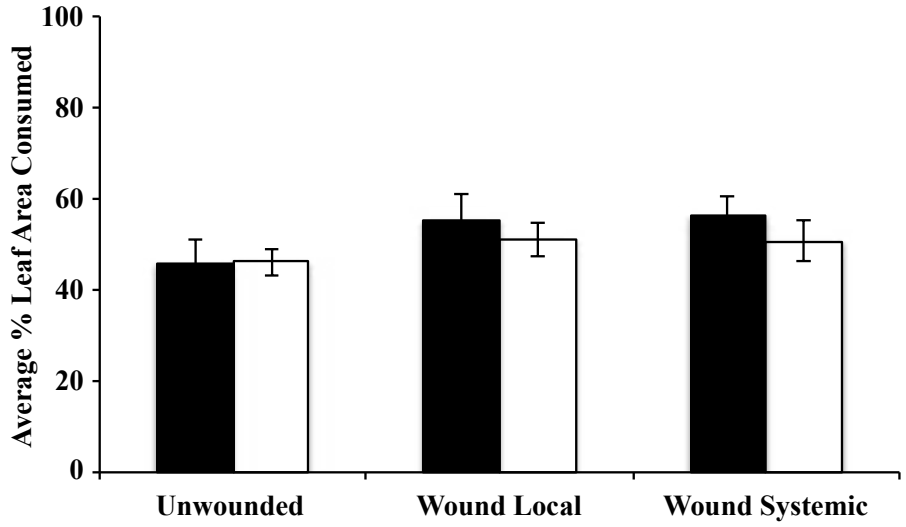

Figure 17: Results for *237p-15* unwounded, wound local, and wound systemic choice bioassays. *237p-15* is shown in white, *Pt x Pa* wildtype is shown in black. WMTM larvae did not show a preference between *237p-15* and *Pt x Pa* wildtype leaves in unwounded, wound local, and wound systemic choice bioassays (Nested ANOVA, $p>0.05$, n=6 pairs. Bars represent mean +/- SE.

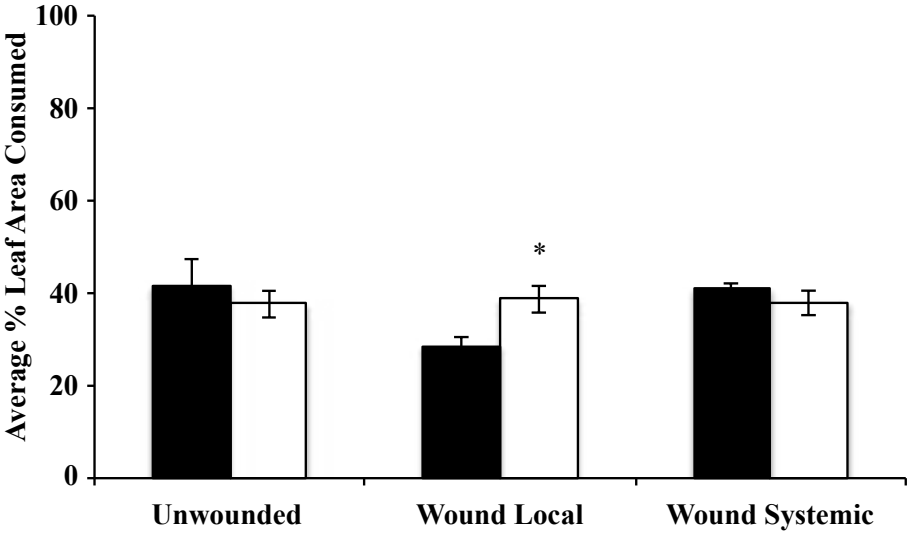

Figure 18: In the first independent set of choice bioassays WMTM larvae preferred to feed on wound local leaves from *239L-4* over *Pt x Pa* wildtype trees. *239L-4* average leaf area consumption is shown in white, *Pt x Pa* wildtype is shown in black. WMTM consumed 30% more leaf area on *239L-4* wound local leaves than *Pt x Pa* leaves (Nested ANOVA, $p<0.005$, n=7 pairs). Bars represent mean +/- SE. * indicates statistical significance.

WMTM larvae consumed 23.7% less leaf area on *239L-4* in unwounded choice bioassays (figure 19; Nested ANOVA, $p<0.05$, n=7 pairs), and 31.5% less area in wound local choice bioassays (figure 19; Nested ANOVA, $p<0.05$, n=7 pairs). WMTM did not show a preference in wound systemic choice bioassays (figure 19; Nested ANOVA, $p>0.05$, n=7 pairs). Because *237p-15* and *239L-4* either did not result in a difference in feeding

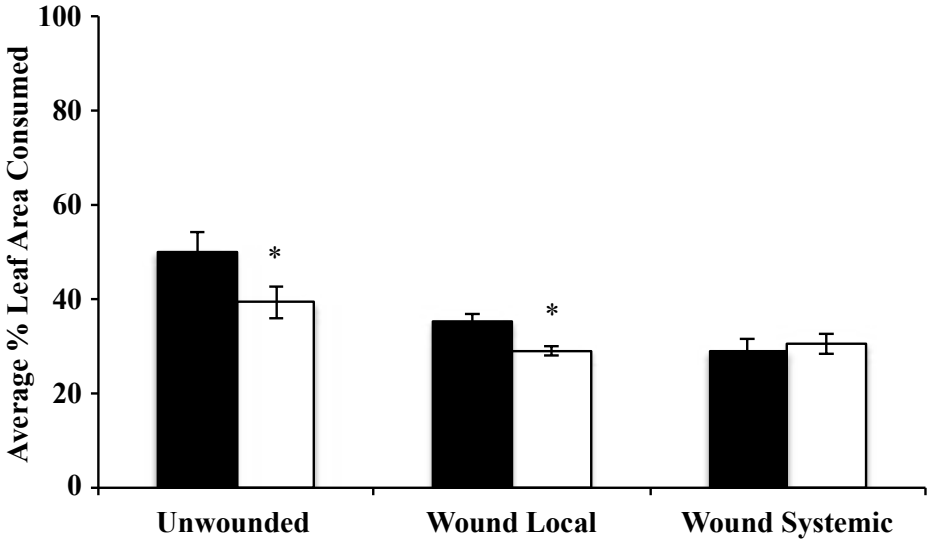

Figure 19: In the second independent set of choice bioassays WMTM larvae preferred to feed on unwounded and wound local leaves from *Pt x Pa* wildtype trees over *239L-4*. *239L-4* average leaf area consumption is shown in white, *Pt x Pa* wildtype is shown in black. WMTM larvae consumed 23.7% less leaf area on *239L-4* in unwounded choice bioassays (Nested ANOVA, $p<0.05$, n=7 pairs), and 31.5% less area in wound local choice bioassays (Nested ANOVA, $p<0.05$, n=7 pairs). Bars represent mean +/- SE. * indicates statistical significance.

preference or had inconsistent results they were ultimately dropped as candidates. The other two mutants showed consistent results from the initial mutant-mutant screen and validation experiments. In the initial comparison against *Pt x Pa* wildtype, *173L-4* was consumed less on unwounded and wound local leaves. When biological replication was increased to nine pairs of trees WMTM preferred to feed more on *Pt x Pa* wildtype leaves

than *173L*-4 leaves in unwounded (31.8%, Nested ANOVA, p=0.0001) and wound local (24.4%, Nested ANOVA, p<0.05) choice bioassays (figure 20).

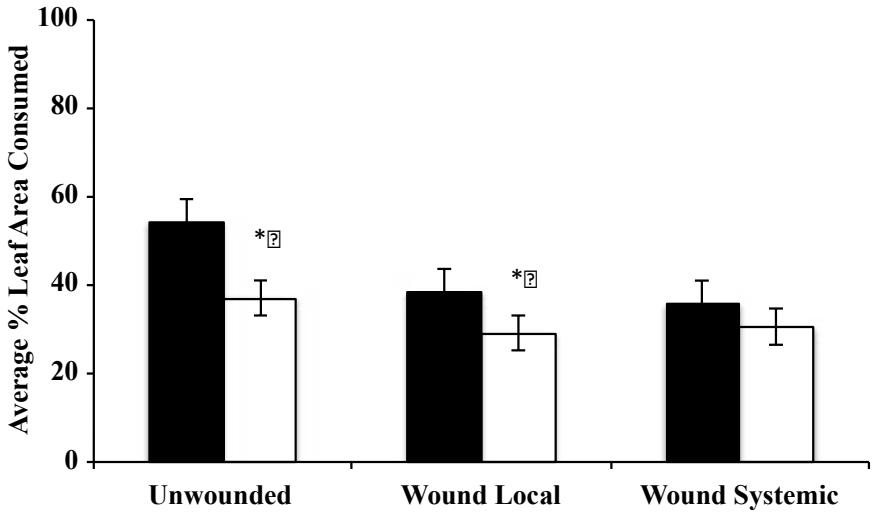

Figure 20: Results for unwounded, wound local, and wound systemic choice bioassays comparing WMTM feeding preference for *173L-4* and *Pt x Pa* wildtype trees. *173L-4* average leaf area consumption is shown in white, *Pt x Pa* wildtype is shown in black. WMTM preferred to feed on *Pt x Pa* wildtypes leaves than *173L*-4 leaves in unwounded (31.8%, Nested ANOVA, p=0.0001) and wound local (24.4%, Nested ANOVA, p<0.05) choice bioassays). * indicates statistical significance. Bars represent mean +/- SE.

Another candidate that we pursued more heavily, *357L-1*, has consistently been preferred by WMTM in choice bioassays. During the mutant-mutant and validation comparison WMTM consumed more leaf weight from *357L-1* in unwounded, wound local, and wound systemic bioassays (figure 21 A-C). In an unwounded choice bioassay using eight biological replicates WMTM consumed 26.2% more leaf area from *357L-1* than *Pt x Pa* wild-type trees in unwounded assays (figure 21A; Nested ANOVA p=0.0206, 26.2% difference). WMTM also showed greater preference for *357L-1* in wound local (figure 21A; Nested ANOVA p<0.0005, 37.4% difference) and wound systemic (figure 21A; Nested ANOVA p<0.00001, 40.6% difference) assays. In on-tree no-choice assays

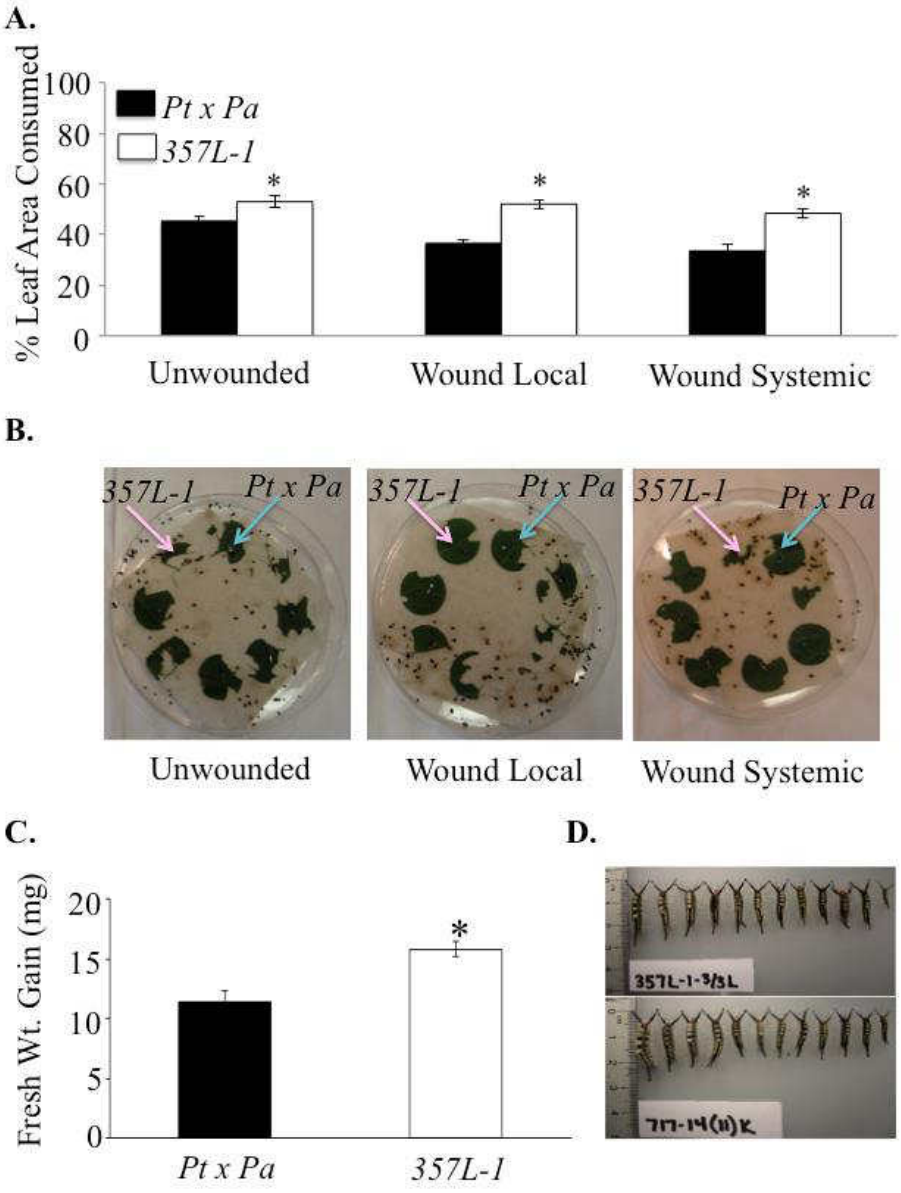

Figure 21: Assays with greater biological replication revealed WMTM preferred feeding on *357L-1* over *Pt x Pa* wildtype leaves in choice assays and greater insect weight gain development in an on-tree no-choice development assay. A) In choice assays with eight biological replicates per genotype WMTM consumed more leaf weight from *357L-1* in unwounded (Nested ANOVA p=0.0206, 26.2% difference), wound local (Nested ANOVA p<0.0005, 37.4% difference) and wound systemic (Nested ANOVA p<0.00001, 40.6% difference) than *Pt x Pa*. Bars represent mean +/- SE. B) Representative plate photos for unwounded, wound local, and wound systemic assays. *357L-1* leaves were was pinned with pink pins, *Pt x Pa* was pinned with blue pins. C) In no-choice development assays WMTM caged on *357L-1* trees gained more weight (15.78 mg) than insects on *Pt x Pa* trees (11.36 mg) over eight days (Two sample *t*-test p<0.003). Bars represent mean +/- SE, n=7 trees per genotype. D) Representative profiles from insects reared on *357L-1* and *Pt x Pa* trees.

WMTM caged on *357L-1* gained more weight (15.78 mg) than insects caged on *Pt x Pa* (11.36 mg) over eight days (figure 21C; Two sample t-test $p<0.003$, n=7 trees per genotype).

These results show that we can use choice bioassays to identify mutants with altered constitutive and induced defenses to WMTM. While we identified 108 (14%) candidates from the initial screen, more than half are likely to be eliminated with further bioassay validation. Half of those candidates will likely be eliminated as they were likely probably paired with a mutant with altered IR. If the trend from the 21 mutants that we selected for validation were to remain true for the rest of the candidates we will be able to eliminate additional false positives. Therefore, the percentage of candidates with altered IR in the population is probably less than five percent. This puts the number of interesting mutations closer to the percentage observed in screens to identify mutants with obvious developmental mutations (Harrison et al. 2007, Busov et al. 2010). This is also a much more manageable number in terms of being able to pursue greenhouse and molecular validation of interesting mutants. Further validation followed by molecular work to identify T-DNA insertions and activated genes on multiple candidates will likely need to be completed on candidates identified from this screen before additional work can be completed to characterize genes involved in IR.

Conclusion

Nine candidates from the QU population were further investigated with follow-up bioassay experiments. From those candidates one mutant, *E8-16*, revealed IR as WMTM preferred to consume *Pt* x *Pa* over *E8-16* leaves and additional no-choice assays revealed that the mutant had a negative impact on insect growth and development. We also

conducted a screen of 770 AT mutants from MTU using unwounded, wound local, and wound systemic choice assays to identify mutants with altered constitutive or induced defenses. We identified several candidates from the Michigan Tech screen that, with further bioassay validation and eventual T-DNA mapping, may prove to be valuable targets for gene identification and characterization. Because we have done more validation experiments with the *E8-16* mutant and they have shown relatively consistent results in all bioassay types performed we ultimately decided to continue with molecular work designed to identify the T-DNA localization, gene identification, and gene characterization of the *E8-16* mutant which will be discussed in the next chapter. Bioassays from the forward genetic screen of AT mutants has identified several possible candidates for further work that may reveal important genes involved in plant defense against insect herbivory.

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CHAPTER IV

IDENTIFICATION AND CHARACTERIZATION OF *10s12800*, A PUTATIVE E3 RING-H2 UBIQUITIN LIGASE OVER-EXPRESSED IN THE *E8-16* ACTIVATION TAGGED MUTANT

Contributions of Authors

Justin Burum performed all air-layer propagation, tree care, and tissue harvesting for all experiments. Justin Burum also performed the pSKI074 vector confirmation PCR experiment. Brett Gross and Dr. Steven Ralph conducted SiteFinding and TAIL PCR T-DNA mapping, qPCR for identifying the "activated gene", and full length cloning of *10s12800* cDNA and generated the gels for those experiments. Dr. Steven Ralph performed the BLAST search and generated the boxshade plot while Justin Burum analyzed the 10s12800 amino acid sequence for functional domains for the protein bioinformatics analysis. Dr. Danquong Huang and Dr. Wenhao (David) Dai created all *10s12800* transgenic and vector control lines. Brett Gross and Dr. Steven Ralph isolated RNA for the microarray and Dr. Matias Kirst and Dr. Cintia Ribiero conducted the microarray hybridization as well as statistical analysis for that experiment. Jen Neva and Dr. Steven Ralph performed the BLAST of significant *Populus* probes from the microarray against the *P. trichocarpa* genome and non-redundant NCBI database and created the microarray results table. Jen Neva and Dr. Steven Ralph generated the RNA

and cDNA for the microarray validation and performed the qPCRs with *10s12800* and *3s16845*. Matthew Flom, with assistance from Justin Burum, generated data for microarray validation for *3s16840* and *3s16845*. Justin Burum generated data for *4s10990* and *11s12420*. Justin Burum, with assistance from Ben Mohr, isolated RNA, created cDNA, and performed the qPCR analysis for the constitutive tissue 10s12800 expression and *10s12800* gene expression experiments. Justin Burum performed the unwounded choice and on-tree no-choice development bioassays for transgenic lines with vector controls.

Introduction

AT has become an effective gain-of-function approach to identify and study gene function related to a phenotype of interest in plants (Weigel et al. 2000; Wan et al. 2009; Busov et al. 2010). In chapter 3 we discussed a forward genetics approach using AT *Populus* that led to the identification of the IR mutant *E8-16.* The ultimate goal of any forward genetics strategy is to identify genes that cause the phenotype of interest. The *E8-16* mutant was created by the Regan Lab (Harrison et al. 2007) by inserting the pSKI074 AT vector (Weigel et al. 2000) into the *Pt x Pa* genome using *Agrobacterium*-mediated transformation (Tzfira et al. 1997; Han et al. 2000). Upon insertion the four CAMV enhancers on the vector can "activate" a nearby endogenous gene causing over-expression (Weigel et al. 2000). Therefore, the first step towards identifying the mutation in *E8-16* required mapping where the pSKI074 T-DNA(s) had inserted. Then, nearby

genes could be tested for expression to identify the activated gene, followed by additional experiments to characterize the gene to understand how the gene may be causing IR.

T-DNA Mapping

One significant advantage of using a T-DNA insertional mutagenesis approach, as opposed to mutations introduced through chemical (e.g., ethyl methanesulphonate) or physical (e.g., x-rays) methods, is the relative ease in which T-DNA locations can be mapped because the DNA sequence is known. The general approach taken towards identifying T-DNA insertion site(s) within AT mutants has been to use the T-DNA vector as a molecular marker in a PCR based strategy to amplify the genomic region flanking the T-DNA or to use plasmid rescue techniques. Early methods required laborious manipulations of the DNA, such as restriction digests and ligations before PCR could be used (Ochman et al. 1993) making it difficult to optimize for more rapid identification of T-DNA inserts within plant genomes.

In 1995 Liu et al. developed a more efficient PCR method called TAIL PCR originally designed to amplify target DNA sequences adjacent to known yeast artificial chromosome (YAC) and PI clones. Liu et al. (1995b) adapted this technique to determine genomic sequences flanking T-DNA insertions within transgenic lines of *Arabidopsis thaliana*. The method used three nested vector specific primers in successive reactions in combination with arbitrary degenerate (AD) primers. AD primers are primer sequences where some of its positions can have several different possible bases. By using AD primers, amplification efficiency for specific products, such as unknown genomic DNA near T-DNA insertion, could be controlled by thermal conditions during PCR (figure 22). The use of semi-nested primers allowed for the amplification of rare sequences, such as a

single T-DNA, within large genomes. Liu et al. (1995b) used specific primers to pGDW32 T-DNA vector along with AD primers to amplify insertion specific products from 183 out of 195 tested *Arabidopsis* T-DNA insertion lines, suggesting that it is an efficient means to identify T-DNA locations.

Another PCR method that has been more recently created is called SiteFinding PCR (Tan et al. 2005). Like the TAIL PCR method, this method incorporates the use of AD and vector specific primers to amplify the genome flanking the inserted T-DNA. In this method, 61 bp oligonucleotides called SiteFinder-1 and SiteFinder-2 contain several random nucleotides along with specific nucleotides at the 3' end to bind to many locations within the plant genome. The oligonucleotide sequences of 5'-NNNNNNGCCT-3' at the 3' end of SiteFinder-1 and 5'-NNNNNNGCGC-3' at the 3' end of SiteFinder-2 are designed to bind to GCCT or GCGC sites within the genome respectively, ideally binding to a location within a few thousand nucleotide base pairs from T-DNA insertion site. On average, each of these four bp sequences should occur once every 256 bp. The long oligonucleotides have sites for three different primers for easy use to amplify PCR products flanking T-DNA and AT vectors when used in combination with vector specific primers (figure 23). If the PCR product is produced via amplification using two SiteFinder primers instead of one T-DNA primer and one SiteFinder primer then the DNA that will form stable stem-loop structures preventing exponential amplification. If, however, the PCR product is produced with both a SiteFinder primer and a T-DNA primer, then amplification will go unhindered during the three rounds of nested PCR. Successful amplification of *Populus* DNA near the T-DNA will result in a bp shift between the secondary and tertiary rounds of PCR using gel

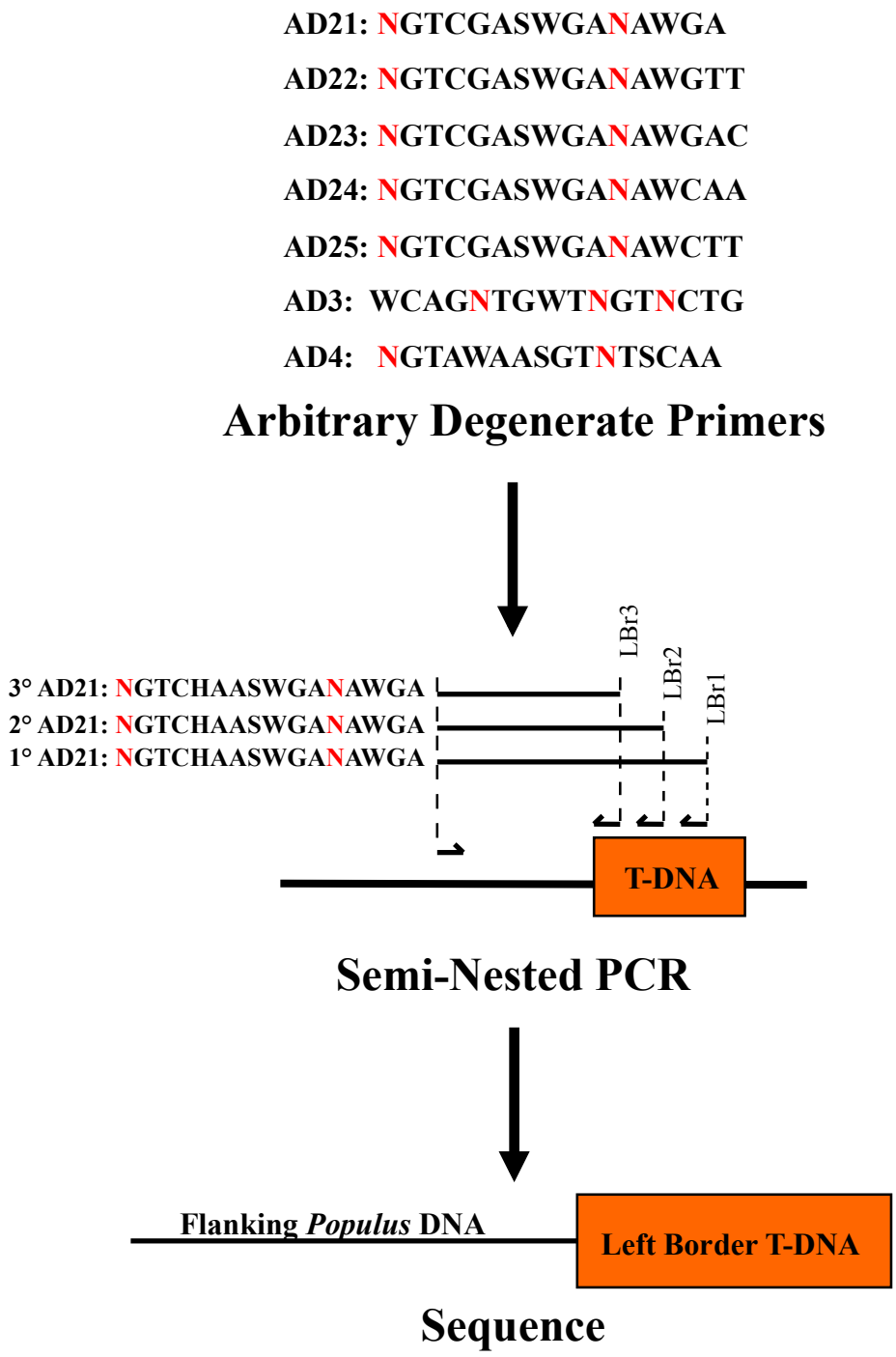

Figure 22: Thermal asymmetric interlaced PCR (TAIL PCR). Seven different arbitrary degenerate (AD) primers used in combination with primers designed to the left border of the AT vector pSKI074 (LBr1, 2, and 3) are used in a three round, semi-nested PCR to amplify *Populus* DNA flanking the vector. PCR products can then be cloned and sequenced. Location can then be determined by alignment with the *Populus trichocarpa* sequence. N= any of the four nucleotides. S=G or C. W=A or T.

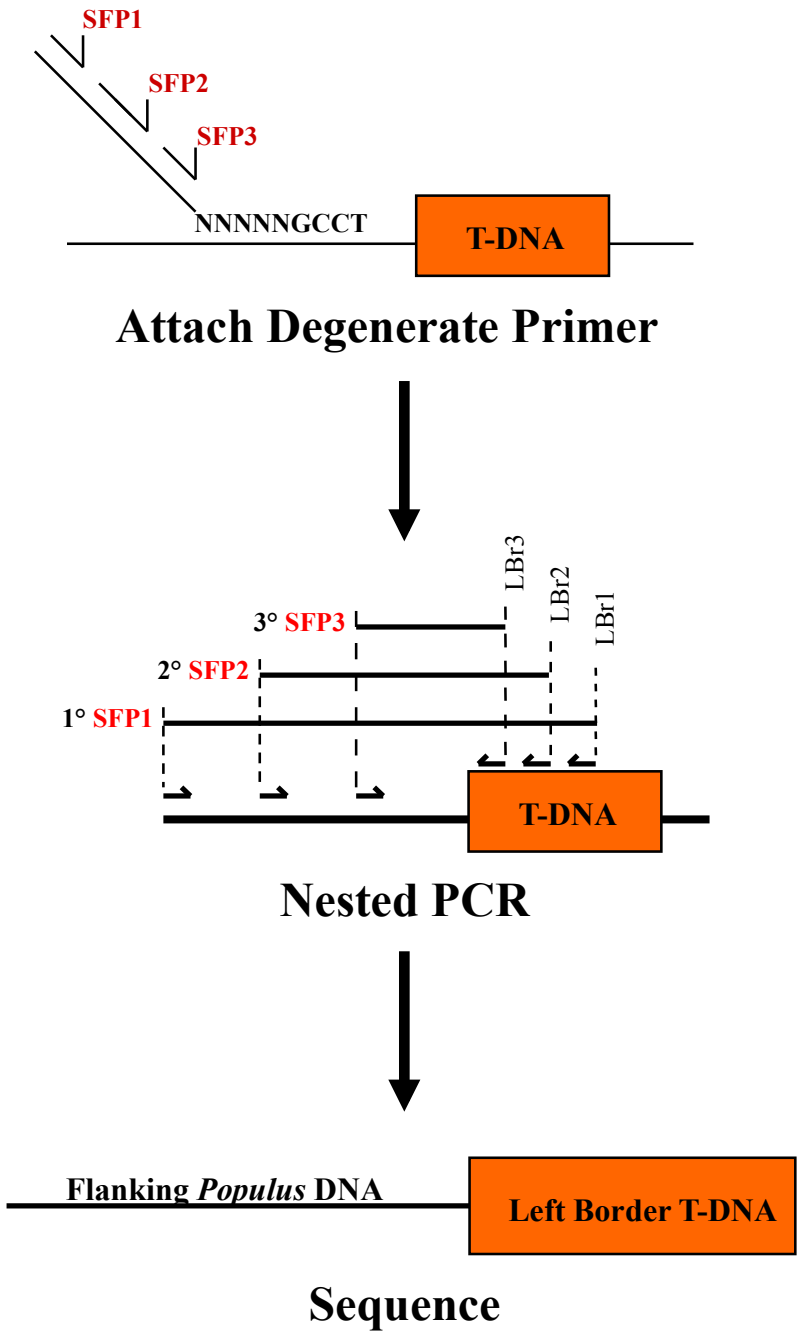

Figure 23: SiteFinding-PCR. In this PCR method long oligonucleotides containing a series of degenerate nucleotides bind to thousands of locations within the *Populus* genome. Using primers designed to the oligonucleotide (SFP1, 2, 3) along with primers designed to the left border of the T-DNA (LBr 1, 2, 3) the method can amplify genomic DNA flanking the T-DNA. PCR products are then cloned and sequenced and a BLAST analysis of the PCR product can reveal T-DNA localization within the *Populus* genome.

electrophoresis. Now that we have discussed PCR methods used to determine T-DNA insertions, it is important to know where other researchers have found AT vectors within mutants and how they identified the activated gene.

Previous Studies of T-DNA Mapping

Weigel et al. (2000) created two AT vectors, pSKI015 and pSKI074 (Figure 24), containing four copies of CaMV enhancers and strategically placed restriction enzyme sites for rescue of sequences from either the right or left border. Vacuum-infiltration was used to generate AT mutants in several *Arabidopsis thaliana* backgrounds (Weigel et al. 2000). In their largest screen (25,000 transformants) they identified and confirmed 23 mutants with altered morphological phenotypes. Several lines were characterized in terms of phenotype as well as for location and identification of activated genes. T-DNA with flanking genomic DNA were recovered by plasmid rescue and analyzed by restriction mapping and DNA sequencing with comparison to GenBank. Genes near the T- DNA sites were tested using qPCR to identify overexpressed genes. What they found was that the distance between the T-DNA and the over-expressed genes ranged from 380 bp to 3.6 Kb. Ichikawa et al. (2003) generated 61,591 independent *Arabidopsis thaliana* lines that were transformed with the AT vector pPCVICEn4HPT. Of those lines 1262 lines (2%) showed an abnormal morphology, growth rate, plant color, flowering time, or fertility in tissue culture or planted in soil. From these lines they isolated and mapped T-DNA insertions within the genome. A total of 1,172 independent T-DNA positions from the 1262 AT mutants (93%) were identified, with 885 inserting outside coding regions. The other 317 mutations were within the reading frame of genes and therefore disrupted gene function. Regions near the centromere had less T-DNA integration and integration tended

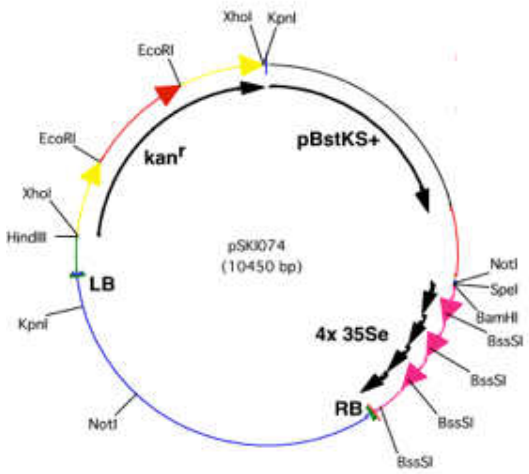

Figure 24: Vector map of pSKI074. This figure shows the orientation and characteristics of the AT vector pSKI074 used by Weigel et al. (2000). This vector was also used to create the *E8-16* mutant.

to cluster in "hot spots" of particular chromosomes. However they did observe relatively even distribution between chromosomes and an average of 2.1 insertions per mutant. Distances between the enhancer and the activated gene varied between 0.7 and 8.2 Kb.

TAIL PCR has been optimized and used to identify AT vector insertion sites within *Populus*. Busov et al. (2010) used AT pSKI015 and pSKI074 vector specific primers and seven different degenerate primers (AD 21, AD 22, AD 23, AD 24, AD 25, AD 4, and AD5) to recover DNA sequences flanking T-DNA insertion sites within 109 AT *Populus* mutants. A total of 136 T-DNA insertions were recovered from 109 mutants, demonstrating that this method is effective in determining AT vector insertions. Most T-DNA inserts (70%) were found within intergenic space and only 13.4% within introns and 15.7% within exons (Busov et al. 2010). The average distance of a T-DNA from a codon start site was 5 Kbp however nearly a third of all insertions were less than half that distance. They also found that the average number of insertions per AT mutant was 1.25,

slightly lower than studies in *Arabidopsis*. TAIL has also been successfully used to identify the location of T-DNA insertions in a variety of plant species with sequenced genomes including *Arabidopsis thaliana* (Liu et al. 2006, Nakazawa et al. 2003), *Oryza sativa* (Mori et al. 2007), and *Lotus japonicas* (Imaizumi et al. 2005).

These studies revealed several characteristics about T-DNA insertions that need to be considered when T-DNA mapping. First, T-DNA insertions can occur between and within gene coding regions. If inserted near an endogenous gene, enhancers may recruit transcriptional machinery resulting in over-expression. Because there is a considerable amount of intergenic DNA in a species' genome it is more likely the T-DNA will insert in those regions for a given AT mutant. If a T-DNA inserts within a gene it may disrupt gene expression or function. Second, these studies indicated that the distribution of inserted T-DNAs for a given AT population was even between chromosomes. These studies suggest that although the T-DNA insertion is more or less random, in roughly 90% of mutants the T-DNA can be identified. Third, multiple T-DNA insertions were common within mutagenic lines. The PCR method(s) chosen for T-DNA mapping needs to be able to identify multiple PCR sites. Finally, these studies also suggest that once the T-DNA insertions are found, genes located nearest the T-DNA site are more likely to be impacted by the enhancers and can be tested using qPCR. These studies provide us a context as what we can expect and confidence that we can apply PCR for T-DNA mapping folluped with qPCR to measure gene expression to accomplish objective #2 of this dissertation.

Objective #2 of Dissertation

Objective #2 of this dissertation was to map the T-DNA insertion(s) and identify the activated gene(s) in the AT mutant *E8-16.* To accomplish this objective SiteFinding-PCR (Tan et al. 2005) and TAIL PCR (Busov et al. 2010) were used to amplify the region of the *Populus* DNA next to the left border of the pSKI074 vector within the *E8-16* mutant. A BLAST analysis of all PCR product sequences against the *Populus trichocarpa* genome (Phytozome, www.phytozome.net) identified a single T-DNA insertion within chromosome 10. Using qPCR we determined that *10s12800*, the gene located closest to the T-DNA insertion, showed a nearly seven-fold increase in expression within *E8-16* leaves compared to the wild-type trees suggesting that *10s12800* expression is activated by the the T-DNA enhancers.

Objective #3 of Dissertation

In addition to identifying the activated gene within *E8-16*, it is also important to understand how the gene is causing the IR phenotype. To characterize the *10s12800* gene, we conducted several different experiments. We performed a basic bioinformatic analysis of the protein by identifying predicted transmembrane and functional domains, and performing a BLASTP analysis within the NCBI non-redundant database (http://blast.ncbi.nlm.nih.gov) to find putative orthologs of the activated gene (*10s12800*) in other plant species. Next, we measured *10s12800* abundance in a tissue panel to determine where the gene was expressed. To understand how the mutation was impacting global gene expression patterns in the *E8-16* mutant, we also examined gene expression using an Agilent 4x44K microarray along with a separate qPCR validation experiment of select genes. Finally, to provide independent genetic confirmation *10s12800* over-expression was causing IR, new transgenic tree lines were created by inserting an

additional copy of the *10s12800* gene in a pCAMBIA S1300 T-DNA vector into two *Populus* hybrid backgrounds. Ultimately we determined that 10s12800 is a putative E3 RING-H2 ubiquitin ligase that contains a really interesting new gene (RING) domain along with two transmembrane domains near the N terminus. The constitutive tissue expression analysis revealed ubiquitous expression but greatest expression in mature leaves. Over-expressing the *10s12800* gene in *E8-16* appears to only impact a limited number of genes in the *Populus* transcriptome including chitinases and MADS-Box transcription factors. A two-fold increase in *10s12800* expression in *Pt x Pa* transgenic trees appears to inhibit WMTM feeding but further work is required to recapitulate the phenotype to conclusively prove *10s12800* is causing IR.

Methods

Confirmation of AT Vector Insertion Within *E8-16*

Five new biological replicates of *E8-16* and *Pt x Pa* wildtype trees were propagated and grown in the Starcher Hall greenhouse to a height of ~100 cm using methods described in chapter 3. LPI 11-13 leaves were harvested, frozen with liquid nitrogen, and then stored in a -80 C freezer for eventual experiments. Genomic DNA was isolated from one gram of leaf material from *E8-16* using a Qiagen DNeasy Plant Mini Kit (Qiagen; Valencia, CA, USA) following the manufacturer's instructions. Genomic DNA was quantified and quality checked using a ND-1000 spectrophotometer (Thermo Scientific; Wilmington, DE, USA). Three sets of PCR reactions were set up to determine which regions of the T-DNA were in the *E8-16* mutant. Primer design was based on southern blot probe design by Harrison et al. (2007) to amplify the vector region before the left border (preLB F1 and R1 primers), after the right border, (pSK F1 and R1

primers), and the kanamycin resistance gene (kanR F1 and R1 primers) of the pSKI074 vector (table 4). Unless noted, all primers were purchased from Integrated DNA

Table 4: Primers used for confirming pSKI074 insertion and identifying the T-DNA location within the *E8-16* mutant. Primers were designed for the left border (preLB), right border (pSK) and kanamycin resistance region (kanR) of the pSKI074 vector to determine if the entire T-DNA inserted within E8-16 (T-DNA confirmation). Thermal assymetric interlaced PCR (TAIL) and SiteFinding PCR primers were used to amplify *Populus* DNA flanking the T-DNA.

Primer	Sequence (5'-3')	Purpose
preLB F1	TGTAGATGTCCGCAGCGTTA	T-DNA confirmation
preLB R1	ATCTAAGCCCCCATTTCCAC	T-DNA confirmation
pSK F1	CTCGGGAGTGCTTGGCATT	T-DNA confirmation
pSK R2	ATCATCCTGTGACGGGAACTTTGG	T-DNA confirmation
kanR F1	GCGTGGCTTTATCTGTCTTTGTATTG	T-DNA confirmation
kanR R1	GGCCTACTTTAATTGCTTCCACTGTTA	T-DNA confirmation
AD21	NGTCGASWGANAWGAA	TAIL PCR
AD22	NGTCGASWGANAWGTT	TAIL PCR
AD23	NGTCGASWGANAWGAC	TAIL PCR
AD24	NGTCGASWGANAWCAA	TAIL PCR
AD25	NGTCGASWGANAWCTT	TAIL PCR
AD3	WCAGNTGWTNGTNCTG	TAIL PCR
AD4	NGTAWAASGTNTSCAA	TAIL PCR
LBr1	AAGCCCCCATTTGGACGTGAATGTAG ACAC	TAIL PCR
LBr2	TTGCTTTCGCCTATAAATACGACGGA TCG	TAIL PCR
LBr3	TAACGCTGCGGACATCTAC	TAIL PCR
SiteFinder1	CACGACACGCTACTCAACACACCACC TCGCACAGCGTCCTCAAGCGGCCGCN NNNNNGCCT	SiteFinding PCR
SiteFinder2	CACGACACGCTACTCAACACACCACC TCGCACAGCGTCCTCAAGCGGCCGCN NNNNNGCGC	SiteFinding PCR
SFP1	CACGACACGCTACTCAACAC	SiteFinding PCR
SFP2	ACTCAACACACCACCTCGCACAGC	SiteFinding PCR
SFP3	CGCACAGCGTCCTCAAGCGGCCGC	SiteFinding PCR

Technologies. Fifteen microliters of master mix [2.5 U Paq 5000 (Agilent Technologies; Santa Clara, CA, USA), 1x Paq Buffer, 0.2 µM forward primer, 0.2 µM reverse primer, 250 µM dNTP, water] plus five µL of *E8-16* DNA (20 ng) were used in each reaction for

a total reaction volume of 20 µl. All PCR reactions, unless indicated, were performed on a DNA Engine Tetrad 2 Peltier Thermal Cycler PCR (Bio-Rad; Hercules, CA, USA). Thermal cycler conditions for preLB were as follows: 95°C for two minutes, 35 cycles of 95°C for 30 seconds, 56°C for 30 seconds, then 72°C for two minutes. PCR conditions for KanR were: 95°C for two minutes, 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, then 72°C for two minutes. PCR conditions for pSK primers were: 95°C for two minutes, 35 cycles of 95°C for 30 secs, 53°C for 30 seconds, and then 72°C for two minutes. PCR products were run on a 1% agarose gel to determine if the appropriate sizes were amplified. All gels, unless noted, were stained with ethidium bromide and imaged using an AutoChemi Gel Documenting System (UVP Bioimaging Systems; Upland, CA, USA).

T-DNA Mapping

TAIL PCR was conducted essentially as described in Busov et al. (2010). Genomic DNA was extracted from 1 gram of *E8-16* leaves harvested as described in the prior section. In the primary PCR, genomic DNA was amplified with the pSKI074 vector specific left border round 1 primer (LBr1) in conjunction with one of seven different AD primers (AD21, AD22, AD23, AD24, AD25, AD3, or AD4, table 4) in 25 microliter reactions with Advantage 2 PCR kit (Clontech; Mountain View, CA, USA) according to the manufacturer's instructions (1x Advantage 2 Polymerase Mix, 1x Advantage 2 Buffer, 200 µM dNTPs, 150 µM LBr1 Primer, 100 ng DNA). Primary PCR reactions were amplified with the thermal conditions indicated in table 5. Primary PCR products were diluted in distilled water (1/10) and 2 µL were added to a master mix (1x Advantage 2 Polymerase Mix, 1x Advantage 2 Buffer, 200 µM dNTPs, 200 µM of LBr2 primer, 1.6

μM degenerate primer) for a total reaction volume of of 25 μL. PCR products were amplified using secondary PCR thermal conditions indicated in table 5. A 1/10 dilution of the secondary PCR product in water was added to a 23 μL master mix of the same composition as the secondary PCR with LBr3 and amplified using tertiary PCR thermal

Table 5: Primary, secondary, and tertiary PCR thermal conditions for TAIL PCR.

Reaction	Cycle #	Thermal Conditions
Primary	1	95°C, 2 min
	5	94°C, 30 sec, 64°C, 1min, 72°C, 2 min 30 sec
	1	94°C, 30 sec, 27°C, 3 min, 72°C, 5 min ramp at 0.3°C/sec
	12	94°C, 30 sec, 64°C, 1 min, 72°C, 2 min 30 sec
		94°C, 30 sec, 64°C, 1 min, 72°C, 2 min 30 sec
		94°C, 30 sec, 44°C, 1 min, 72°C, 2 min 30 sec
	1	72°C, 5 min
Secondary	12	94°C, 30 sec, 64°C, 1 min, 72°C, 2 min 30 sec
		94°C, 30 sec, 64°C, 1 min, 72°C, 2 min 30 sec
		94°C, 30 sec, 44°C, 1 min, 72°C, 2 min 30 sec
	1	72°C, 5 min
Tertiary	31	94°C, 30 sec, 50°C, 1 min, 72°C, 2 min 30sec
	1	72°C, 5 min

conditions (table 5). Secondary and tertiary PCR products were were separated by electrophoresis on a 1% agarose gel, imaged, and bands that showed a size shift between the two rounds were excised from the gel using a razor blade and purified using a Gel Extraction Kit per instructions (Qiagen; Valencia, CA, USA). Tertiary PCR products from AD 22, 23, 3 were then ligated into the pCR 4 -TOPO vector and transformed into One Shot TOP10 chemically competent *E.coli* according to the manufacturer's instructions for the TOPO TA Cloning kit (ThermoFisher Scientific; Minneapolis, MN, USA) or directly sequenced. Transformed cells were plated on 1.5 % LB agar plates with 100 μg/mL ampicillin, 80 μg/mL X-Gal (G Biosciences; St. Louis, MO, USA) and 50 μM IPTG (G Biosciences; St. Louis, MO, USA) and grown overnight for blue-white colony

selection.

White colonies were diluted in ten microliters of distilled water in 0.2 µL PCR tubes. Colony PCR using M13F (5'-GTAAAACGACGGCCAGT-3') and M13R (5'-GTTTTCCCAGTCACGAC-3') sequencing primers were used to identify plasmids with the expected insert size based on TAIL tertiary bands. Twenty µL total reactions (Paq5000 2U, 1x Paq Buffer, 200 pMol M13 F primer, 200 pMol M13 R primer, 200 µM dNTP mix, 1 µL of DNA from white colonies) were amplified at the following thermal conditions: 95°C for 2 minutes, 32 cycles of 95°C for 20 seconds, 55°C for 20 seconds, 72°C for 2 minutes, and then 72°C for five minutes. Colony PCRs were run on a 1% gel, stained with ethidium bromide, and imaged. Colonies with a PCR band size matching the TAIL tertiary PCR were grown overnight in 2 mL of LB broth with 200 ng of ampicillin in a shaking incubator at 225 rpm at 37°C. Plasmids were isolated using a Qiagen QIAprep Spin Miniprep kit (Qiagen; Valencia, CA, USA) and then sequenced using an ABI BigDye Terminator 3.1 Cycle Sequencing kit (Thermo Fisher Scientific; Minneapolis, MN, USA) with M13 primers. Sequences were run on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems; Thermo Fisher Scientific; Minneapolis, MN, USA) with the program ABI Sequence Scanner v1.0. Sequences were trimmed using BioEdit software (www.bioedit.software.informer.com) to remove the pCR 4-TOPO vector sequence. A BLASTN analysis of sequences was conducted against the NCBI non-redundant database (www.ncbi.nlm.nih.gov) to determine if products contained a fragment of the pSKI074 T-DNA and against the *P. trichocarpa* genome sequence (http://www.phytozome.net; Goodstein et al. 2012) to determine the T-DNA location within the *E8-16* genome.

SiteFinding-PCR was conducted essentially as described in Yordanov et al. (2010). Sitefinder-1 and 2 primers were annealed to *E8-16* genomic DNA in separate 25 µL reactions (1x Advantage 2 Polymerase Mix, 1x Advantage 2 Buffer, 250 µM dNTP, 10 pMoles SiteFinder-1 or 2 Primer, 100 ng of *E8-16* DNA) at the following PCR conditions: 92°C for two minutes, 95°C for 1 minute, 25°C for 1 minute, and 1 ten minute cycle where temperature was ramped up to 68°C at 0.2°C/sec. Following the annealing of the SiteFinder primers, five µL (50 pMoles SiteFinding primer, 10 pMoles LBr1 primer, 1x Advantage 2 Buffer) were added to the reactions before they underwent the primary PCR. Thermocycler conditions for the primary PCR were: 94°C for 1 minute, 30 cycles of 95°C for ten seconds, 68°C for six minutes, and then 1 cycle at 72°C for five minutes. PCR products were diluted in distilled water (1/100) and then 1 µL was added to a master mix (1x Advantage 2 Polymerase, 1x Advantage 2 Buffer, 25 µM dNTP mix, 0.2 µM LBr2 primer, 0.2 µM SFP2 primer) for a total of 50 µL. Secondary thermal PCR conditions were the same as the primary PCR. A 1/100 dilution of the secondary PCR was used for the tertiary PCR using the same PCR reagents with LBr3 and SFP3 primers with the same thermal conditions as the primary PCR. PCR products from Sitefinder-1 and 2 were ligated into pCR 4-TOPO vector and transformed into TOP10 *E.coli* cells or directly sequenced using methods discussed for the TAIL PCR to determine if products contained a fragment of the pSKI074 T-DNA and to determine the T-DNA location within the *E8-16* genome.

Expression of Genes Near T-DNA Location

Absolute transcript abundance of predicted genes located within 20 kb of the T-DNA were tested using quantitative PCR and normalized to the housekeeping gene

translation initiation factor 5 alpha (*TIF5α)* to identify the activated gene. Total RNA was isolated from LPI 11-13 leaves harvested from three *Pt x Pa* and three *E8-16* trees described in the vector confirmation section, using Qiagen's RNeasy Plant Miniprep kit (Qiagen; Valencia, CA, USA). RNA was quality checked and quantified with a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA.) and Bio-Rad Experion Automated Electrophoresis System (Bio-Rad; Hercules, CA, USA). One microgram of RNA from each tree was treated with DNase1 (Thermo Fisher Scientific; Minneapolis, MN, USA) and cDNA synthesis was generated using an iScript cDNA synthesis kit (Bio-Rad; Hercules, CA, USA) according to manufacturer's instructions. Three genes located near the T-DNA site were tested for gene expression. POPTR_0010s12800 (*10s12800*) was amplified using primers 5'-GAGATGATGCATGTGCATGATG-3' and 5'-TGTAGCAGCTAAGAAATTCCAAG-3'. Primers for amplification of POPTR_0010s12810 (*10s12810*) were 5'-CTCCTTCGCTAGGTAAACTCTC-3' and 5'- ATTCCGATACTGACAAGTTGTTC-3'. POPTR_0010s12790 (*10s12790*) was amplified using the primers 5'-CAGCATTAATCAAACTAGTACTCC-3' and 5'-CATCTAATGCTATAACTTCTCAGC-3'. TIF5α was amplified using the primer set 5' -GACGGTATTTTAGCTATGGAATTG-3' and 5'-CTGATAACACAAGTTCCCTGC-3'. PCRs were conducted in 25 μL reactions (1x Advantage 2 Polymerase, 1x Advantage Buffer, 0.2 μM of the forward and reverse primer, 0.2 mM dNTPs, 1 μL of 50 ng of *Pt x Pa* or *E8-16* DNA). PCR conditions for amplifying all three genes were: 95°C for two minutes then 30 cycles of 95°C for 15 seconds, 56°C for 15 seconds, 68°C for one minute, then 68°C for 15 minutes. PCR products were separated on a 1.5% agarose gel to

ensure amplification occurred at the appropriate size. PCR products were purified using a QIAquick PCR Purification kit (Qiagen; Valencia, CA, USA), ligated into the pCR4-TOPO vector and transformed into TOP10 chemically competent *E.coli* as described above, and then sequenced using ABI BigDye Terminator 3.1 Cycle Sequencing kit (Thermo Fisher Scientific; Minneapolis, MN, USA). pCR4-TOPO plasmid standards for each gene were prepared for calculating absolute gene expression. qPCR runs consisted of a 10-fold dilution series for plasmid standards ($6x10^{-2}$ to $6x10^{-9}$ ng), 25 ng of cDNA for all trees, and RNA and water negative controls using iQ SYBR Green Supermix (Bio-Rad; Hercules, CA, USA) at the following conditions: 95°C for 15 minutes, then 40 cycles of 94°C for 10 seconds 60°C for thirty seconds 72°C for 30 seconds followed by a plate read, then 72°C for ten minutes, and then a melting curve between 65°C to 95°C with readings every 0.2 seconds followed by 72°C for ten minutes. All qPCR runs were run on a Chromo4 AlphaBlock Assembly Detector (Bio-Rad; Hercules, CA, USA). Four technical replicate qPCR runs were made for each gene to ensure technical reproducibility. Amount of plasmid per ng of of RNA was calculated based on the standard curve and normalized to *TIF5α* for each gene. Molecular weights of pCR 4-TOPO standards with gene inserts were calculated according to Invitrogen (http://www.invitrogen.com/site/us/en/home/references/Ambion-Tech-Support/rna-tools-and-calculators/dna-and-rna-molecular-weights-and-conversions.html) to calculate copy number.

Protein Bioinformatics

10s12800 is predicted by Phytozome to have a coding sequence of 517 bp, translating to 169 amino acids. To determine the class of protein we conducted a

BLASTP search of the non-redundant database analysis of the National Center for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) database. Homologues that were found to be similar (<e-10) were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2; Larkin et al. 2007). A boxshade plot was created (BOXSHADE v.3.21, https://sourceforge.net/projects/boxshade/) to identify conserved regions within the group of proteins. To identify transmembrane helices within 10s12800 we used the ARAMEMNON database (http://aramemnon-botanik.uni-koeln.de) which integrates predictions from 18 individual programs (Schwacke et al. 2003). InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan; Quevillon et al. 2005) and PROSITE (http://prosite.expasy.org; Sigrist et al. 2010) were used to identify functional domains.

Constitutive Expression of *10s12800*

To determine where the *10s12800* gene is expressed within *Pt x Pa* tissues a separate greenhouse experiment was conducted. Five *E8-16* trees and four *Pt x Pa* trees were propagated from original stems from QU using air-layering and then grown in three-gallon pots in the Starcher Hall greenhouse as described in chapter 3. Trees were grown to ~180 cm, with a 3:1 ratio of Sunshine Mix #1 and sand, under a 16:8 day:night cycle with supplemental lighting, with water and fertilizer provided *ad libitum.* From four of the *E8-16* and *Pt x Pa* wildtype trees we harvested mature leaves (LPI 11-13), immature leaves (LPI 0-2 and crown), bark, xylem, phloem, petioles, and roots tips using sterile razor blades. Tissues were immediately frozen in liquid nitrogen, and then stored at -80°C. For analyzing constitutive expression of *10s1280,* RNA was isolated from *E8-16* and *Pt x Pa* tissues using the Qiagen RNeasy Plant Miniprep kit (Qiagen; Valencia,

CA, USA) and quality checked and converted to cDNA with an iScript cDNA Synthesis kit (Bio-Rad; Hercules, CA, USA) as previously described. Four technical qPCR runs for each RNA sample were performed using 25ng of cDNA using the same *10s12800* and *TIF5α* primers used to determine the activated gene. Absolute gene expression, normalized to *TIF5α*, was done as previously described above in the section for determining gene expression near the T-DNA.

Microarray Analysis

To determine global expression differences between *E8-16* and *Pt x Pa* wildtype trees a microarray analysis was conducted. Mature LPI 11-13 leaves that were previously harvested for the constitutive tissue panel were also used to compare global transcriptomic expression differences between *E8-16* and *Pt x Pa* trees. One gram of RNA was isolated from five *E8-16* and four *Pt x Pa* trees using the Qiagen RNeasy Plant Minikit (Qiagen; Valencia, CA, USA) per the manufacturers instructions. RNA was quantified and quality checked as previously described. RNA was then shipped to the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida where microarray hybridization and image processing was performed. cDNA synthesis and Cy3 and Cy5 dye labeling was performed using an Agilent Low-Input Quick Amp Labeling kit (Agilent; Santa Clara, CA, USA). The *Populus* microarray was generated using Agilent's SurePrint technology and consisted of gene probes arranged in a 4x44K format per slide. The microarray comprised one 60-mer probe per gene for 61,361 previously described gene models derived from the annotation of the genome sequence of *P. trichocarpa* clone 'Nisqually-1' (version 2.2; Tuskan et al. 2006) and a set of non-

annotated ESTs supported with transcriptional evidence. Duel hybridizations and image analysis were performed at the ICBR as described in Drost et al. (2009).

To confirm the results from the microarray we propagated and grew five new *E8-16* and *Pt x Pa* trees within the greenhouse and then individually tested genes using qPCR. POPTR_0010s12800 (*10s12800*, E3 ubiquitin ligase), POPTR_003s16840 (*3s16840*, MADS-box), POPTR_003s16845 (*3s16845*, MADS-box), POPTR_004s10990 (*4s10990*, bZIP transcription factor), and POPTR_0011s12420 (*11s12420*, NAM protein) were selected as probes because these genes were found to be over-expressed in *E8-16* from the microarray. Another gene, POPTR_008s12460 (*8s12460*, E3 ligase) that has amino acid sequence similarity to *10s12800* was also tested for absolute gene expression relative to *TIF5α*. Primers were designed to either the 3' UTR or between two exons for all genes. The primers used for *10s12800* were the same as those used in the initial identification of the activated gene. *3s16840* was amplified with the primers 5'-GAGGCGAAATGGATTGTTCAAG-3' and 5'-CTCGCGTATAAGCAACGATAAG-3'. *3s16845* was amplified with the primers 5'- CGGTAAAGACTCGGATCACCC-3' and 5'- CCTCCAGCGGTAAGTTATCAC-3'. The bZIP transcription factor *4s10990* was amplified with the primers: 5'- CCTCCTCAGCTTCAAGAATTC-3' and 5'-GTGAGCACTTGACGTAGTTCAG-3'. The NAM protein 11s12420 was amplified with the primers 5'- CTTCTCAGTCAACTTCCACAG-3' and 5'-CTACTAATCAATCGACGACCG-3'. Genes were amplified with the same reagents and conditions as previously described followed by agarose gel confirmation, sequencing, and cloning into pGEM T-Easy for amplification standards, and then qPCR.

Full-length *10s12800* cDNA

An important step in proving whether or not the *10s12800* gene is causing the IR phenotype observed in the *E8-16* genotype requires recapitulating the phenotype by creating new transgenics intentionally over-expressing the gene by inserting an extra copy of *10s12800* gene within a wildtype background. The first step towards recapitulation required amplifying the full-length of *10s12800* gene so that it can be cloned within a suitable vector for *Agrobacterium* transformation into the *Populus* genome. One forward primer (F2 5'-ATCGCATAACCTAAAACACTG-3') with one of two different reverse primers (R1 5'-TGTAGCAGCTAAGAAATTCCAAG-3', R2 5'-GAATGCACTCTTATAGCAG-3') designed to the 5' and 3' untranslated regions (UTR) of the predicted gene model POPTR_10s12800 were used to amplify *10s12800*. Twenty µL reactions (1x Advantage 2 Polymerase Mix, 1x Advantage 2 Buffer, 200 µM dNTPs, 0.2 µM F2 primer, 0.2 µM R1 or R2 primer, 8.34 ng cDNA from *E8-16*) were used to amplify full length *10s12800* cDNA with the following PCR conditions: 1 cycle 95°C for two minutes and 32 cycles of 95°C for 15 seconds, variable temperatures for 30 seconds, 72°C for 1 minute. Using cDNA from one of the *Pt x Pa* trees used for the qPCR gene expression analysis we attempted to amplify the gene using a temperature gradient with three different annealing temperatures: 51.7, 55.5, or 61.8°C. PCR products were loaded and separated on a 1% agarose gel. The single intense band generated using the F1-R2 primer combination at the 55.5°C annealing temperature was PCR purified using a Qiagen PCR Purification Kit and cloned into the PCR4-TOPO vector as previously described. Twenty µL colony PCR reactions (2U Paq 5000, 1x Paq Buffer, 200 pMol F primer, 200 pMol R primer, 200 µM dNTPS, 1 µL of bacteria in water) using M13F and M13R primers were conducted using the following PCR thermal cycler conditions: 1

cycle 95°C for two mins, 32 cycles of 95°C for 20 seconds, 55°C for 20 seconds, 72°C for one minute, and one cycle at 72°C for five minutes. Colonies were run on a gel and colonies with bands at approximately 517 bp were grown overnight in 1 mL LB broth with amplicillan on a shaking incubator at 37°C. Plasmids were purified using a Qiagen Plasmid miniprep kit and then sequenced to confirm that the colonies contained the full sequence of the *10s12800* gene without mutations.

Generation of *10s12800* Transgenic Trees

10s12800 cDNA was amplified using primers with *Xba I* and *Sac I* restriction sites (5'- GTATCTTCTAGAAAAAATGGGTTTGCAAAACCAG -3', 5'-CATAGAGAGCTCTCATCTCAAGGAGAGCCAG -3'). PCR amplification was performed according to manufacturers instruction for the Elongase Enzyme Mix (Invitrogen; Carlsbad, CA, USA). PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen; Valencia, CA, USA) and then cloned into the pGEM-T Easy vector (Promega; Madison, WI, USA) and transformed into chemically competent cells. Bacteria was plated on LB broth with ampicillin and grown overnight at 37°C. Positive colonies with the insert were selected (blue-white colony selection) and confirmed using colony PCR (figure 25). Select plasmids were grown overnight, isolated, and sequenced. *10s12800* cDNA from pGEM-T Easy was cut and inserted into the multi-cloning (MC) site of the plant expression vector pCAMBIA S1300 next to the vector's super promoter (a trimer of octopine synthase activator, monopine synthase activator, and mas promoter) as shown in figure 25. *10s12800* constructs were transfected into *Agrobacterium tumefasciens* strain *EHA105* and PCR was used to confirm that the correct size of *10s12800* (517bp) was transformed into the *Agrobacterium tumefasciens EHA105*

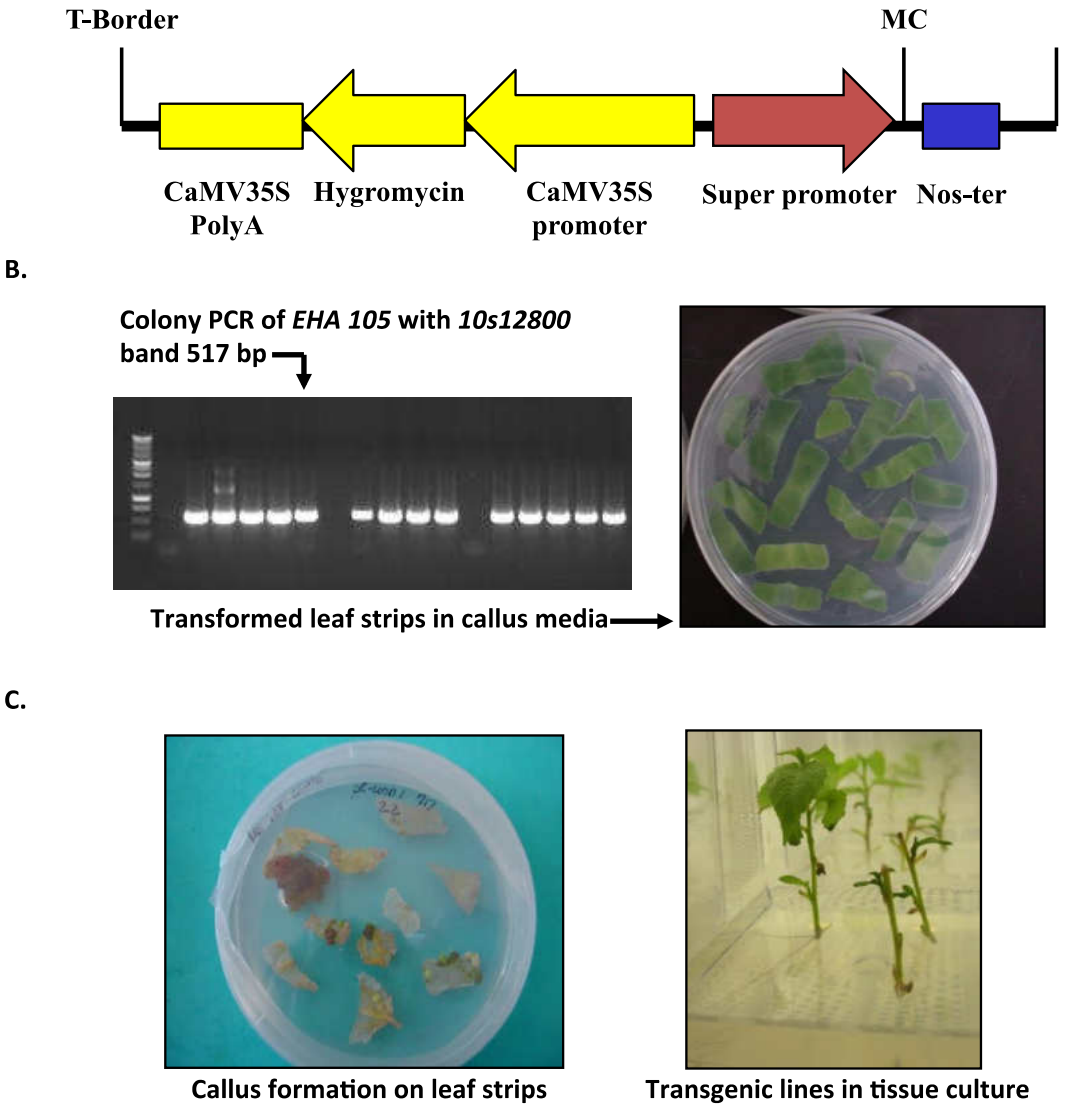

Figure 25: Generation of *10s12800* transgenic lines. A) Full length 10s12800 cDNA was inserted in the pCambia S1300 vector in the multi-cloning site. B) cDNA with vector was transformed with *Agrobacterium* and a colony PCR was performed to identify bacteria with the correct insert size (517 bp). Leaf tissue was exposed to transformed Agrobacterium and placed on callus induction media. C) Callus tissue from transformed cells growing on callus induction plate. After placing callus on root and shoot induction media copies of each independent lines were made in tissue culture.

followed by sequencing. Leaves from *Pt x Pa* and *Pc x Pg* were cut into 0.5-0.8 cm wide pieces and then incubated in a petri plate with *10s12800* transfected *Agrobacterium* for 15 minutes. Explants were then placed on petri plates with co-cultivation media (MS with

5 mM Zip, 10 mM NAA, and 100 mM acetosyringone) and placed in petri plates for two days at room temperature in the dark. Explants were gently washed with autoclaved water and gently dried before being placed on callus induction media (MS with 5 mM Zip, 10 mM NAA, 500 mg/L carbenicillan, 250 mg/L cefotaxime, and 5 mg/L hygromycin) for four weeks in the dark at room temperature. Explants were then transferred to shoot induction media (MS with 0.2 mM TDZ, 500 mg/L carbenicillin, 250 mg/L cefotaxime, 5 mg/L hygromycin) under lights with a 16 hr photoperiod for four weeks. Individual shoots were then placed on rooting media (1/2 MS with 0.5 mM NAA, 500 mg/L carbenicillin, 250 mg/L cefotaxime, and 5 mg/L hygromycin. DNA was extracted from roots of each tree line and a standard PCR was conducted to ensure that each line contained the transgene. Trees lines were propagated in tissue culture and eventually transferred to soil and grown in the greenhouse to a height of >10cm.

qPCR Screen of *10s12800* Trangenic Lines

LPI 2-3 leaves were harvested from five trees from all *10s12800* transgene and vector control line trees using sterile razor blades. Harvested leaves were stored in 50 mL falcon tubes with liquid nitrogen and then stored at -80°C for later use. Total RNA was isolated, quality checked with a Nanodrop UV spectrophotometer, treated with DNaseI, and converted to cDNA as previously described. Primers designed for *TIF5α* and *10s12800* based on the *P. trichocarpa* genome sequence were used to measure absolute expression of *10s12800*. pGEM T-Easy plasmid standards for each gene (i.e., containing the target PCR amplicon) were cloned, sequenced, and quantified by Nanodrop. Each qPCR run consisted of a plasmid dilution series from $6x10^{-3}$ to $6x10^{-8}$ ng, 25 ng of each

cDNA , 25 ng of *Pt x Pa* or *Pc x Pg* RNA, 25 ng of transgene RNA, and water alone. qPCR reactions were performed using iQ SYBR green supermix as previously described.

Bioassays for Select *10s12800* Transgenic Trees

For unwounded choice bioassays eight biological replicates of *10s12800* transgene and vector control *Pt x Pa* trees were grown together and LPI 11-13 leaves were harvested, cut into 6.5cm^2 disks, and pinned on four petri plates per pair (technical replicates). For details of how plates were set up see chapter 3. Six 3rd instar WMTM larvae were placed on each plate and allowed to feed for 24-48 hours. Leaf area was analyzed using GIMP imaging software, and then the leaves were dried and weighed.

On-tree no-choice bioassays to compare insect growth and development between *TL4* transgene and *TL8 Pt x Pa* vector control was conducted as described earlier. Twelve 2nd instar WMTM larvae were caged with mesh bags on LPI 8-16 leaves of ten trees of each line. Insects were reared for seven days on trees until the largest insects were late 4th instar. Insects were removed from each tree and weighed for fresh weight gain.

Statistical Analysis

Statistical analysis of all bioassay data and gene transcript abundance data was conducted in R (www.r-project.org). Leaf consumption for choice bioassays, transcript abundance of genes located near the T-DNA, *10s12800* constitutive expression, and the microarray transcript expression validation were analyzed using a nested-ANOVA test. Wet and dry weight gain of insects were analyzed using a two-sample t-test and instar development was analyzed using a chi-square test. Constitutive tissue expression was analyzed using a one-way ANOVA followed by a post hoc Tukey's HSD test. For the microarray raw signal data from all hybridizations were quantile-normalized, log2-

transformed and analyzed using a gene-by-gene mixed ANOVA model in SAS 9.2. Data was filtered for background noise based on negative control spots on the microarray, and probes displaying low signal (<200 units) were considered below the detection threshold.F-tests were performed for genotype effect and least-square mean estimates were obtained analyzed using q-value at false discovery rate (FDR) of 0.15.

Results

T-DNA Confirmation and Localization

Before additional work was conducted to identify the T-DNA localization we tested whether or not the mutant *E8-16* contained a T-DNA. PCR primers were used to amplify the left border (preLB), kanamyacin resistance (kanR), and right border regions (pSK) of the psKI074 vector. The left border and kanR regions amplified with the expected products at 160 bp (lane 5) and 454 bp (lane 10) respectively (figure 26). Weak amplification at ~1431bp with pSK primers (lane15) suggests that the right border of the T-DNA may be degraded from endonucleases during the *Agrobacterium* transformation into the *Populus* tissue.

To determine the T-DNA location(s) within the *E8-16* mutant we used two PCR methods, TAIL PCR and SiteFinding-PCR. For TAIL PCR we used seven different degenerate primers along with pSKI074 vector specific products to amplify *E8-16* genomic DNA flanking T-DNA insertions. If primers specifically amplify the genomic region flanking a T-DNA we would expect to see a 50bp shift between the secondary and tertiary rounds of PCR. Of the seven degenerate primers tested, five (AD 21, 22, 25, 3, 4) showed an approximate 50bp band shift between the secondary and tertiary rounds of PCR (figure 27). We also observed non-specific products in each PCR. Substantial non-

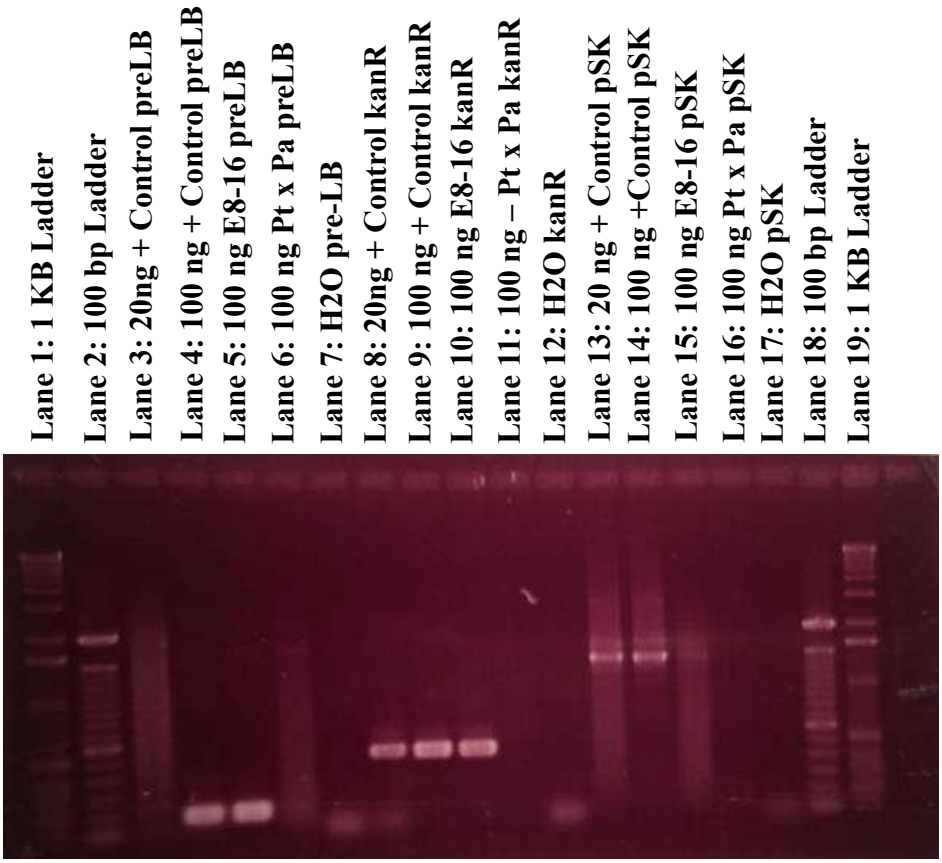

Figure 26: T-DNA confirmation of the pSKI074 vector within *E8-16*. A) Three sections of the AT vector were amplified: the left border (pre-LB), kanamycin region (KanR), and the right border (pSK). The preLB and kanR products amplified strongly at the same expected bp sizes as the positive control. For the pSK primer set there was weak amplification at the expected size of 1431 bp. Gel was run with a positive control, another AT mutant called *R19-6*, at two different DNA amounts, along with *Pt x Pa* and water negative controls.

specific background products can be generated using this method since two AD can amplify DNA without the pSKI074-specific primer. We also found PCR products in water negative controls for several of the AD primers. Contamination, which was non-specific, is common when performing multiple rounds of semi-nested PCR. We chose to gel excise, clone, and sequence the products from AD 22, 3, and 4 as they had PCR product bands that did not correspond with bands in the negative controls for those AD

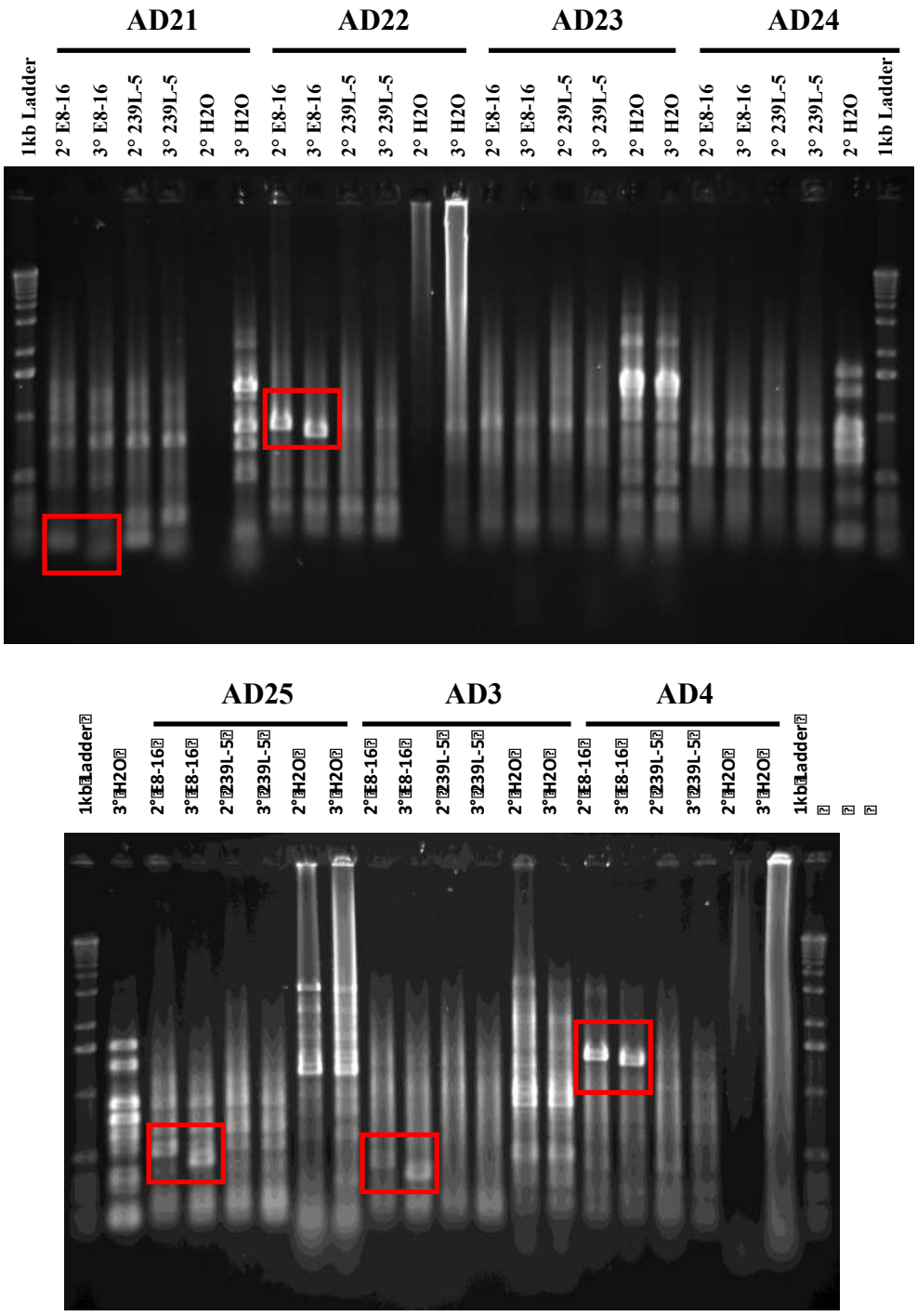

Figure 27: Results for *E8-16* using TAIL PCR. Three rounds of semi-nested TAIL PCR were used to amplify *Populus* DNA flanking the T-DNA insertion site with pSKI074 vector specific primers and AD primers (AD21, 22, 23, 24, 25, 3, 4). Gels show the secondary and tertiary PCR products for all seven AD primers for *E8-16* and water negative controls, as well as an additional AT mutant *239L-5* that was being investigated at the same time. Five of the AD primers showed the characteristic 50 bp band shift between PCR rounds (highlighted in red box).

primers.

Following gel excision, tertiary products from AD 22 and AD 4 were directly sequenced. We were unable to directly sequence the tertiary product from AD 3 but we were able to clone the product into the pCR4-TOPO vector and purify the plasmid for sequencing. Vector sequences were trimmed using BioEdit to remove the T-DNA sequence and then another BLAST analysis was done in Phytozome against the *Populus trichocarpa* genome. AD3 mapped to the12786 kb position on chromosome 10 (E value=2.3E-51) with a 95.5% identity match (106/110 bp). AD 4 (E value=2.6E-44) and AD22 (E value=1.1E-49) also had the strongest match to the same location on chromosome 10 with an identity score of 96.4% (106/110 bp) and 94.5% (121/128 bp), respectively.

SiteFinder-1 and 2 products that specifically amplify the genomic region containing the T-DNA should reveal a 70 bp band shift between PCR rounds. SiteFinder-1 revealed a single PCR band at ~1500bp in the secondary PCR and two bands at ~1400 bp and 400 bp in the tertiary (figure 28). With the SiteFinder-2 primer we observed an intense band at ~1550bp in the secondary PCR that appeared to shift 70 bp in length in the tertiary PCR. We also observed several other minor bands. We attempted to directly sequence the clone the two products from the tertiary of SiteFinder-1 and the intense bands from the secondary and tertiary rounds of SiteFinder-2. A BLAST analysis from the 1400 bp band from SiteFinder-1 and the 1550 bp from the secondary PCR of SiteFinder-2 within the non-redundant database of NCBI revealed the sequence of pSKI074 activation-tagged vector and *Populus* DNA. BLAST analysis of the tertiary PCR product from SiteFinder-1 against the *P. trichocarpa* genome in Phytozome had the

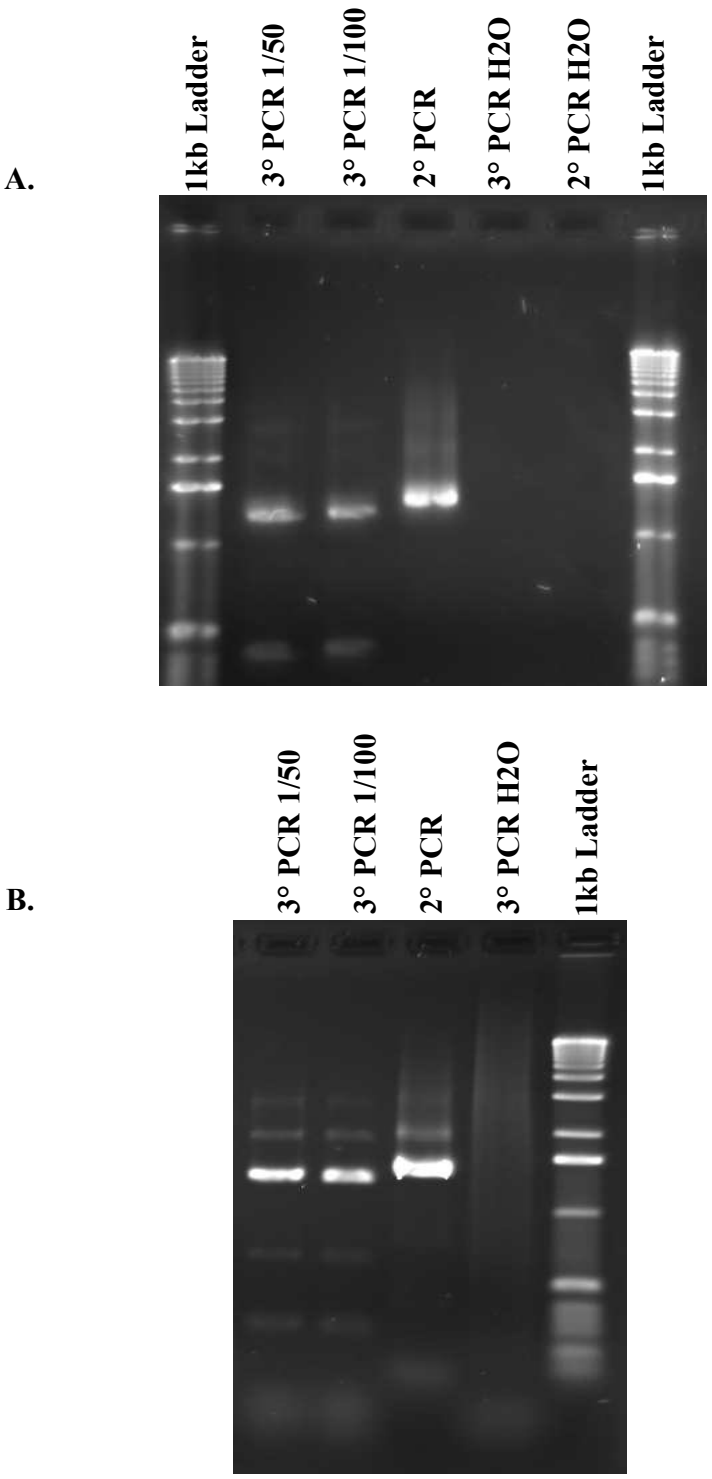

Figure 28: SiteFinding-PCR results for *E8-16* using SiteFinder-1 and SiteFinder-2 oligonucleotides. A) SiteFinder-1 revealed a single intense band at ~1500 bp in the secondary PCR and two products in the tertiary (~1400 bp and ~400bp). B) SiteFinder-2 showed an intense band ~1550 bp in the secondary PCR and multiple products in the tertiary but the most intense band at ~1480 bp.

strongest match at chromosome 10 (E=1.8E-51) with an identity of 95.3% (122/128 bp). We were unable to sequence the tertiary product from SiteFinder-2 but the secondary PCR product had the strongest match at 12786 kbp on chromosome 10 (E=1.2E-83) with an identity of 95.9% (185/193 bp).

qPCR Expression Analysis of Predicted Gene Models Near the T-DNA

To determine if there was transcriptional activation near the T-DNA we selected three gene models located near the insertion site for expression analysis using qPCR. Two predicted gene models, *10s12800* and *10s12810*, were selected because most activated genes are within 10 kb of the insertion site (Busov et al. 2010). We also chose to test the predicted gene model *10s12790* as the CAMV enhancers are orientated to impact genes on either side of the T-DNA. *10s12810*, a predicted serine-threonine kinase, was not detectable in leaf tissue. Expression of *10s12800*, a predicted E3 ubiquitin ligase, was 6.94 fold higher in *E8-16* leaves than *Pt x Pa* (figure 30; Nested ANOVA, $p<0.00001$). Expression of *10s12790*, a predicted fructose-bisphosphate aldolase, was 1.53 fold higher in *E8-16* leaves than *Pt x Pa* (Nested ANOVA, $p=0.0104$). *10s12800* shows a pattern of 5-10 fold higher expression in AT vs. wild-type lines with strong statistical support. These results suggest *10s12800* is the activated gene in the *E8-16* mutant.

10s12800 is a Putative E3 RING-H2 Ubiquitin Ligase

A BLASTP analysis was performed to identify all available homologues with sequence similarity (e-10). We then compared sequences using a Clustal W2 alignment of proteins to identify to identify conserved regions. The BLASTP comparison of the

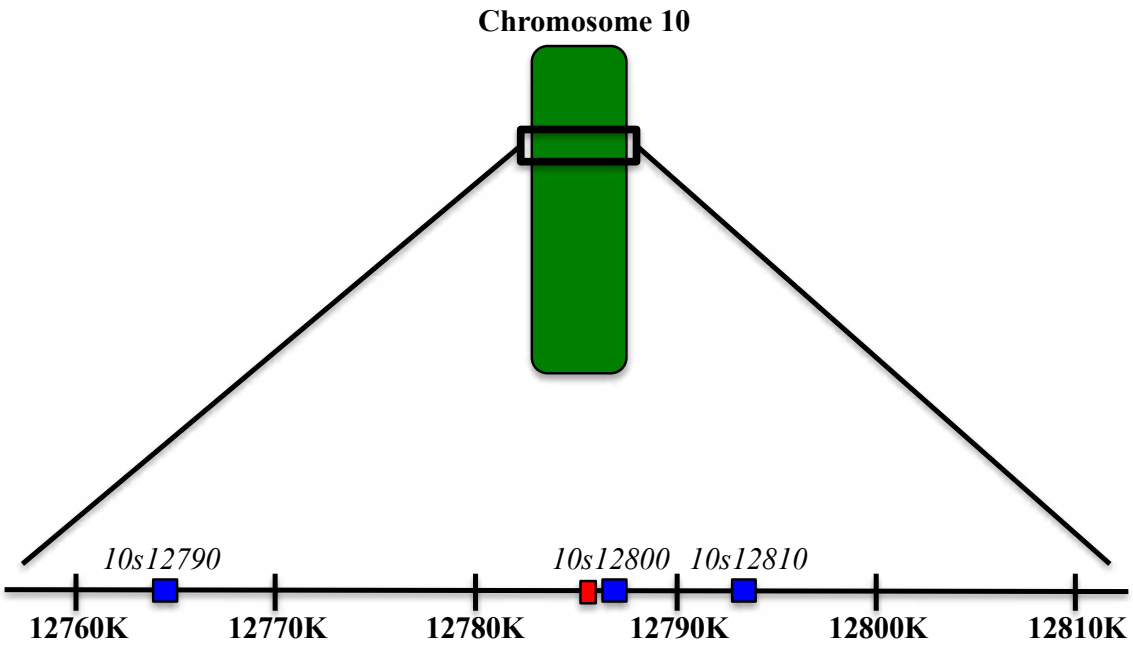

Figure 29: SiteFinding and TAIL PCR results for identifying a T-DNA insertion within the *E8-16* mutant. SiteFinding and TAIL PCR identified the same location on chromosome 10 as shown in the red box. Three gene models are located in close proximity to the T-DNA, *10s12790*, *10s12800*, and *10s12810* (blue boxes).

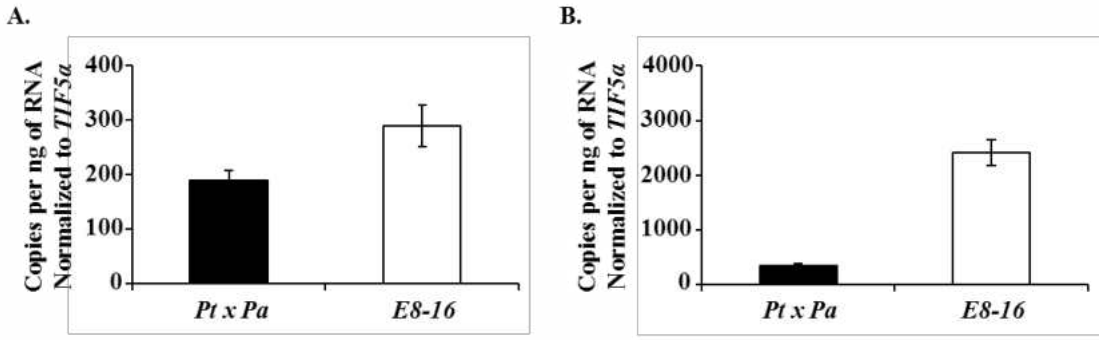

Figure 30: Absolute transcript expression of *10s12790* and *10s12800*. A) Expression of *10s12790* was 1.53 fold higher in *E8-16* leaves than *Pt x Pa* (Nested ANOVA, p=0.0104). Expression of *10s12800*, a predicted E3 ubiquitin ligase, was 6.94 fold higher in *E8-16* leaves than *Pt x Pa* (Nested ANOVA, p<0.00001). Bars represent mean +/- SE.

10s12800 amino acid sequence against the non-redundant database from GenBank

identified proteins with similar sequences found throughout the plant kingdom including

Orzyia sativa, *Arabidopsis*, and *Populus* (Figure 29). Two regions of *10s12800* (1-44 and 100-148 amino acid location 2) showed strong sequence similarity to other proteins which prompted us to further investigate whether these areas corresponded with known functional domains or secondary protein structures. InterProScan and PROSITE confirmed the presence of a really interesting new gene (RING) zinc finger domain between amino acids 100-142. InterProScan identified two transmembrane domains at amino acid positions 15-35 and 55-71 amino acids while the ARAMEMNON plant membrane protein database suggested that the two transmembrane domains are between amino acids 13-33 and 50-70. PROSITE also confirmed the presence of a zinc finger RING type profile from amino acids 100-142. Another look at the RING domain in the boxshade plot revealed a conserved Cys3His2Cys3 motif suggesting that *10s12800* is an E3 RING-H2 ubiquitin ligase that appears to be localized within a membrane. The ARAMEMNON database suggested that the protein is likely involved in the secretory pathway and does not predict an N-terminal cleavable signal peptide. There is a mix in results about whether the N-terminus is facing the cytosol.

***10s12800* Constitutive Expression**

cDNA from seven tissues (bark, immature leaves, phloem, roots, mature leaves, xylem, and petioles) from four *Pt x Pa* wildtype trees were tested for *10s12800* expression normalized to *TIF5α*. As shown in figure 32, *10s12800* expression was observed in all tissues tested. A one-way ANOVA showed that the effect of tissue type on *10s12800* expression was significant, ($F_{6,21}$=10.51, p=0.00002). A post hoc Tukey HSD test revealed that mature leave tissue expression differed from petioles, bark, immature leaves, phloem, and roots at $p<0.01$; expression from xylem tissue was not

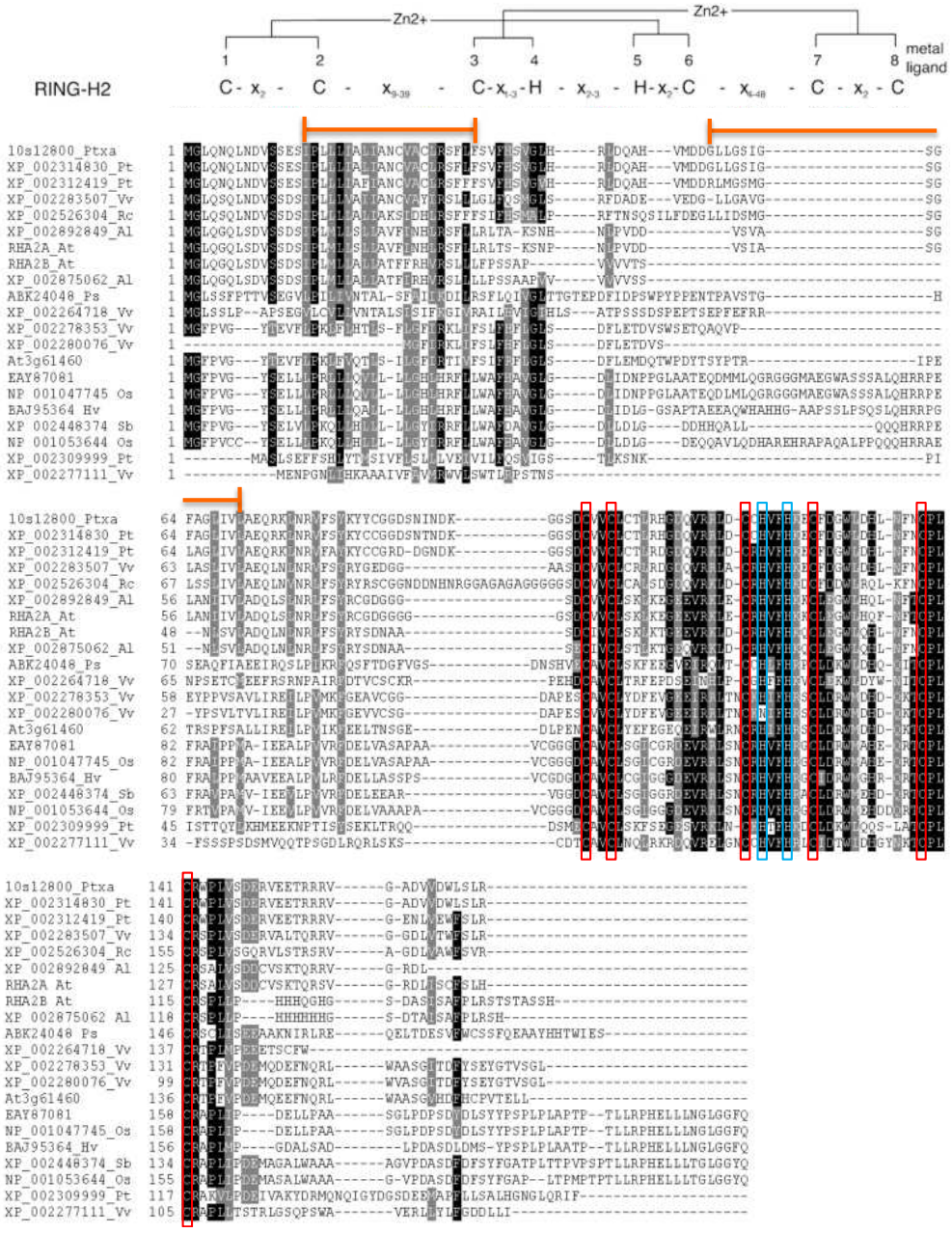

Figure 31: Boxshade CLUSTAL W2 alignment of the 10s12800 protein and 20 related plant proteins identified during a BLASTP search in GenBank. The 10s12800 protein contains two transmembrane domains (indicated in orange) and conserved Cys3: His2: Cys3 residues characteristic of the RING-H2 domain. Black boxes indicate > 60% identity and gray boxes indicate >60% similarity. Conserved cysteine and histidine residues for the RING-H2 domain are indicated with red and blue boxes respectively.

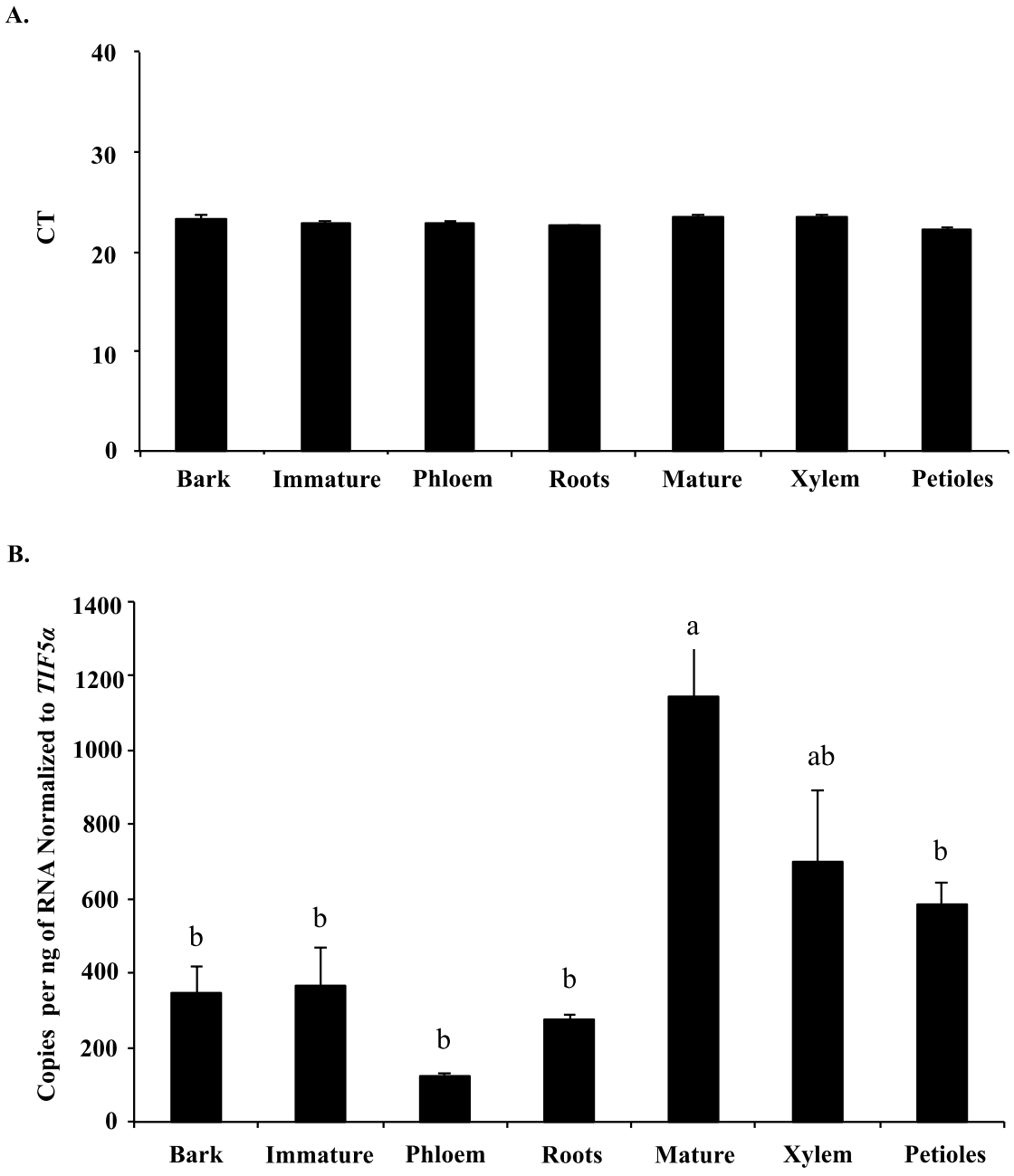

Figure 32: Constitutive expression of *10s12800* in *Populus* tissues. A) *TIF5α* CT range across all tissues was 1.2. B) *10s12800* gene expression was observed in all tissues. A post hoc Tukey HSD statistical test revealed a difference in gene expression between mature leaves and bark, immature, phloem, roots, and petioles ($p<0.01$). Bars indicate mean +/- SE.

significantly different from the two groups, lying somewhere in the middle.

Microarray Comparison and Validation

To compare global transcript level differences between the *E8-16* and *Pt x Pa* wildtype genotypes we conducted a microarray experiment using RNA from LPI 11-13 leaves from four *E8-16* and four *Pt x Pa* trees. Before probes were identified overall signal intensity and slide-to-slide intensity was evaluated with a scatter plot to ensure quality hybridization (figure 33). Slide to slide comparisons revealed correlation coefficients greater than 0.9, suggesting the intensity of probes was even between slides and trees (figure 33). Gene expression for the microarrays was considered statistically significant if p value < 0.05 and a false discovery q value < 0.15. Only 22 probes out of a

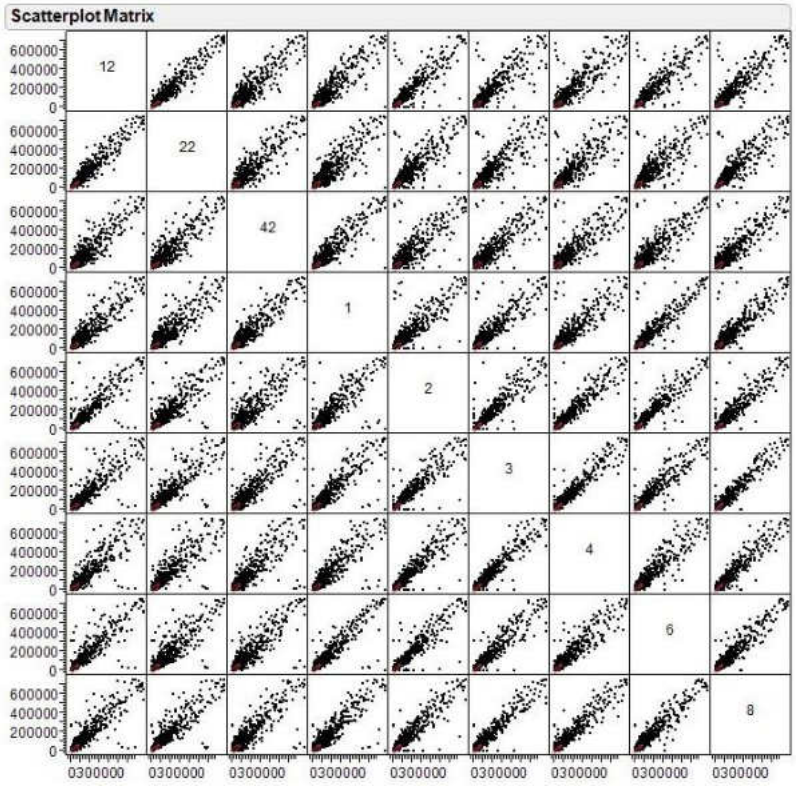

Figure 33: Scatterplot analysis. This graph shows a scatterplot comparison of Cy3 and Cy5 hybridization signal intensities for each slide. Multivariate analysis shows correlation coefficients range from 0.90 to 0.97 across all slides indicating consistent hybridization throughout the experiment and very little noise among microarrays Microarrays with *Pt x Pa* wildtype RNA are 12, 22, 42, 6, and 8. Microarrays with *E8-16* are 1-4.

potential 43,803 revealed expression differences between genotypes (Table 6). *10s12800* was among the most highly differentially expressed genes at levels 6.06 fold higher in *E8-16* versus *Pt x Pa* leaves. PCR experiments had identified this gene as activated by the T-DNA mutation in *E8-16*. Thus, the microarray experiment provides independent confirmation that *10s12800* is the activated gene in *E8-16*. Other than *10s12800* and the probe designed for the pSKI074 vector, probes for MADS-box transcription factors, a chitinase 1, No Apical Meristem (NAM), metallotheionin 3, and a bZIP transcription factor also showed overexpression in *E8-16* compared to *Pt x Pa*. Nine probes for a plant invertase/pectin methylesterase inhibitor, alpha/beta hydrolases, a transferase, a serine-threonine kinase, a class IV chitinase, and remorin were downregulated in E8-16 leaves.

Five new *E8-16* and *Pt x Pa* trees were grown in an independent greenhouse experiment to validate the microarray results. Five genes were chosen for the qPCR validation based on magnitude of expression differencesand statistical support in the microarray experiment. *10s12800*, the RING-H2 ubiquitin ligase, *3s16840* and *3s16845*, predicted MADS-box transcription factors, 4s10990, a predicted bZIP transcription factor, and *11s12420*, a predicted NAM protein, were selected as they were shown to be over-expressed in the *E8-16* mutant in the microarray experiment. We also chose to test *8s12460*, another predicted E3 ligase, as it was the closest related amino acid sequence (60%) identity to *10s12800* in the *Populus* genome. *10s12800* expression was 8.83 fold higher in *E8-16* leaves than in *Pt x Pa* wildtype leaves (figure 34; Nested ANOVA, $p<0.00001$, n=5 pairs). *3s16840* and *3s16845* expression was significantly higher, 14.22 and 23.12 fold higher respectively, in *E8-16* leaves than *Pt x Pa* (figure 34; $p<0.0001$).

Table 6: Twenty-two differentially expressed probes with *Populus* genome ID and annotation along with p and q value scores.

Poplar Genome ID	Poplar Genome Annotation (Phytozome database)	Best BLASTp Hit in *Arabidopsis*	Log_2 Fold Change	p Value	q Value
None	None	Vector Sequences	6.8	<0.0001	0.116
10s12800	E3 Ubiquitin Ligase	At2g01150 (5.00E-27)	2.6	<0.0001	0.116
3s16845	MADS-BOX Protein	At1g77080 (7.00E-07)	3.8	<0.0001	0.116
3s16830, 3s16860	MADS-BOX Protein, None	At1g77080 (8.00E-08), At1g77081 (3.00E-06)	3.2	<0.0001	0.116
3s16840, 3s16870	MADS-BOX Protein	At5g10140 (3.00E-24)	2.6	<0.0001	0.116
3s14640, 3s16870	None, MADS-BOX Protein	At4g24130 (2.00E-69), At5g10140 (3.00E-24)	2.5	<0.0001	0.116
None	None	None	1.0	<0.0001	0.116
1s11920	None	None	-1.4	<0.0001	0.122
6s13740	Plant Invertase/Pectin Methylesterase Inhibitor	At2g45220 (9.00E-134)	-1.4	<0.0001	0.122
5s07540	Alpha/beta Hydrolase	At4g39955 (1.00E-134)	-1.1	<0.0001	0.122
9s14430, 9s14400, 9s14390, 9s14410	Chitinase Class I	At3g12500 (1.00E-131, 7.00E-119, 2.00E-116, 2.00E-122)	1.4	<0.0001	0.122
11s12420	No Apical Meristem (NAM)	At3g15510 (2.00E-115)	1.6	<0.0001	0.122
11s11960	Metallotheionein 3	At3g15353 (1.00E-17)	1.9	<0.0001	0.122
17s12890	Alpha/beta Hydrolase	At5g39220 (2.00E-132)	-1.2	<0.0001	0.122
2s17160	None	At3g61730 (4.00E-43)	-2.9	<0.0001	0.122
None	None	None	2.6	<0.0001	0.122
5s25250	Transferase Family Protein	At1g27620 (0)	-1.0	0.0001	0.122
17s14140	Serine-Threonine Protein Kinase	At5g38560 (0)	-1.0	0.0001	0.122
4s10990	bZIP Transcription Factor	At3g30530 (5.00E-45)	0.8	0.0001	0.122
19s12360	Chitinase Class IV	At3g54420 (3.00E-132)	-1.7	0.0001	0.122
14s02780	Remorin, C-terminal Region	At1g45207 (3.00E-171)	-0.8	0.0001	0.122
2s12390	None	At2g23270 (5.00E-03)	1.4	0.0001	0.122

4s10990 expression was 4.65 fold higher in *E8-16* leaves (figure 34; $p<0.001$) than *Pt x Pa*. Expression for the predicted NAM was 2.9 fold higher in *E8-16* leaves than in *Pt x Pa* ($p<0.0001$). Expression for *8s12460* was 2.36 fold lower in *E8-16* versus *Pt x Pa*

leaves ($p<0.001$). Expression from the validation experiment was consistent with the microarray, although the fold difference for most genes was higher for the qPCR than the microarray.

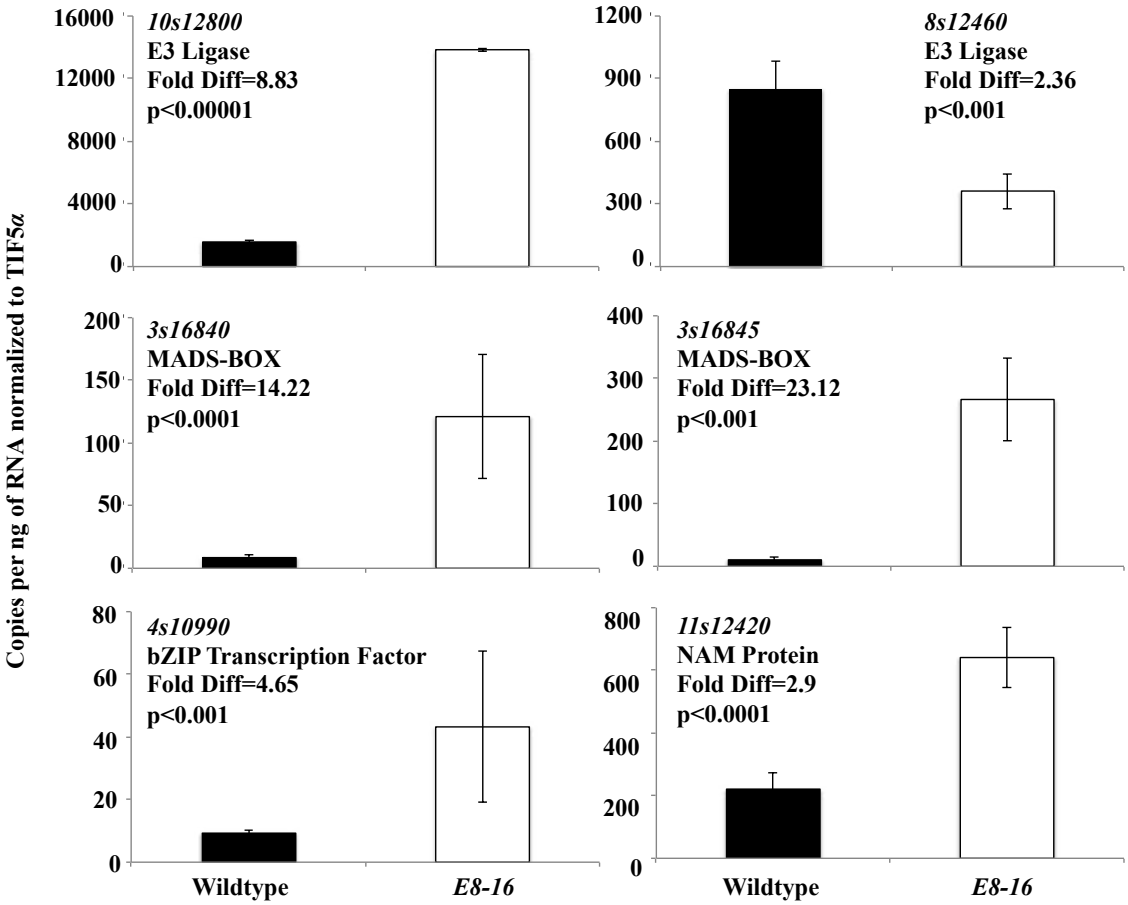

Figure 34: qPCR validation of the microarray. Six genes were selected and tested for gene expression between *E8-16* and *Pt x Pa* leaves. Five genes, *10s12800*, *3s16845*, *4s10990*, and *11s12420* showed significantly higher expression in *E8-16* leaves (Nested ANOVA, $p<0.001$, $n=5$ pairs). *8s12460* revealed 2.36 fold less expression in *E8-16* leaves compared to Pt x Pa (Nested ANOVA, $p<0.001$, $n=5$ pairs). Bars represent SE.

FL-10s12800

The purpose of producing the full-length cDNA of *10s12800* was to use the cDNA to create new transgenic *Pt x Pa* lines over-expressing *10s12800*. To capture the

full-length of the cDNA we designed primers to the 5' and 3' UTRs based on POPTR_10s12800 gene model from the *P. trichocarpa* genome in the Phytozome database, as well as available EST data from the non-redundant database from NCBI. We used one forward primer and two different reverse primers along with three different annealing temperatures in the PCR to successfully capture the sequence without acquiring mutations. As shown in Figure 35, the F1-R2 primer combination at

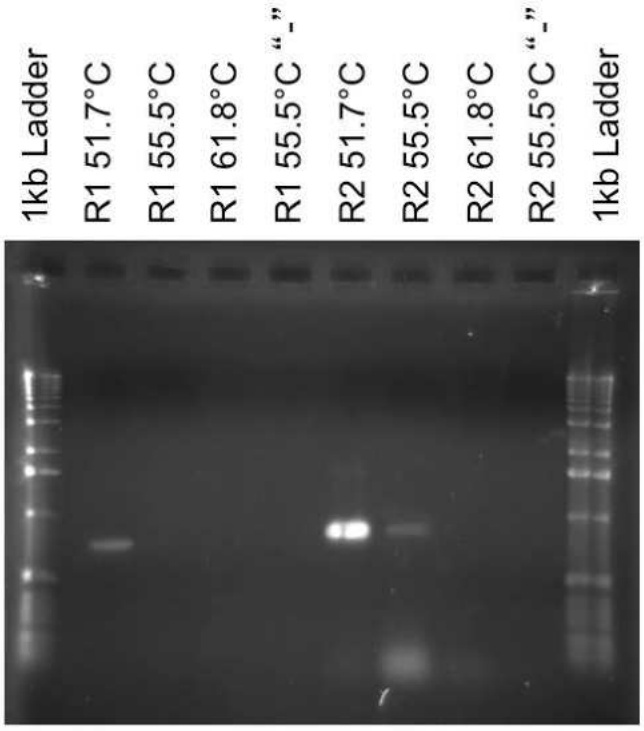

Figure 35: PCR products using forward and reverse primer combinations at different annealing temperatures to amplify the full-length cDNA of *10s12800*. Annealing temperature 51.7°C produced a strong band that was ultimately sequenced.

the 51.7°C annealing tempterature showed a strong PCR band at the expected size of 517 bp. The PCR product was cloned within pCR4-TOPO vector followed by colony PCR and sequencing. We ultimately sequenced three colonies. Colony 1 had the correct

product without mutations based on the *P. trichocarpa* genome sequence. The plasmid was ultimately sent to our collaborators at NDSU to produce the new transgenic trees.

qPCR Screening and Bioassay Validation of *10s12800* Transgenic Lines

Our collaborators at NDSU created 13 transgenic lines within the *Pt x Pa* genetic background and 9 transgenic lines within the *Pc x Pg* background. Trees were brought back to UND and grown in the greenhouse until replicates were >10 cm in height. RNA isolation, cDNA synthesis, and qPCR were done in three batches for each genetic background. Five biological replicates for each *10s12800* transgenic line (TL) as well as vector control lines (VC) were used in our qPCR analysis to identify transgenic lines over-expressing *10s12800*. Our selection criteria was that TL lines had to be over-expressing *10s12800* at least two fold higher than the VC line and have a nested ANOVA $p<0.05$. Only one transgenic line, *TL4* in the *Pt x Pa* background, was found to be two fold higher in expression than *VC8* (figure 36; Nested ANOVA, $p<0.05$, $n=5$). Surprisingly, several lines had lower expression than the VC in the *Pt x Pa* and *Pc x Pg* (figure 37) background. We feel confident that our qPCR results are accurate as the *TIF5α* values were within one cycle threshold (CT) in each batch. In addition, RNA negative and water negative controls only had sporadic non-target amplification after 35 cycles.

Unwounded choice bioassays were used to compare WMTM larvae feeding preference between for three TL lines against vector controls. *TL4* was compared to *VC8* to see if a two-fold increase in *10s12800* expression would result in feeding preference difference. *TL10* and *21* were compared against vector controls (*VC5* and *VC1*) to confirm that mutants that did not have higher expression of *10s12800* did no have an IR phenotype. WMTM did not prefer to feed on *VC5* over *TL21* (figure 38A; Nested

ANOVA, p>0.05, n=8 pairs). In a choice assay between *TL1* vector and *TL10* transgene lines WMTM consumed more leaf material from the control was this was not a statistically significant difference (figure 38B; Nested ANOVA, p>0.05, n=8 pairs). WMTM did consume more leaf area (27%) from the *VC8* compared to the *TL4* transgenic line (figure 38C; Nested ANOVA, p<0.05, n=8 pairs). The leaf area consumption difference was smaller than the original unwounded bioassays with *E8-16*. These results suggest that a two-fold *10s12800* expression difference does result in a small feeding preference difference. Smaller differences in expression, or no difference in *10s12800*

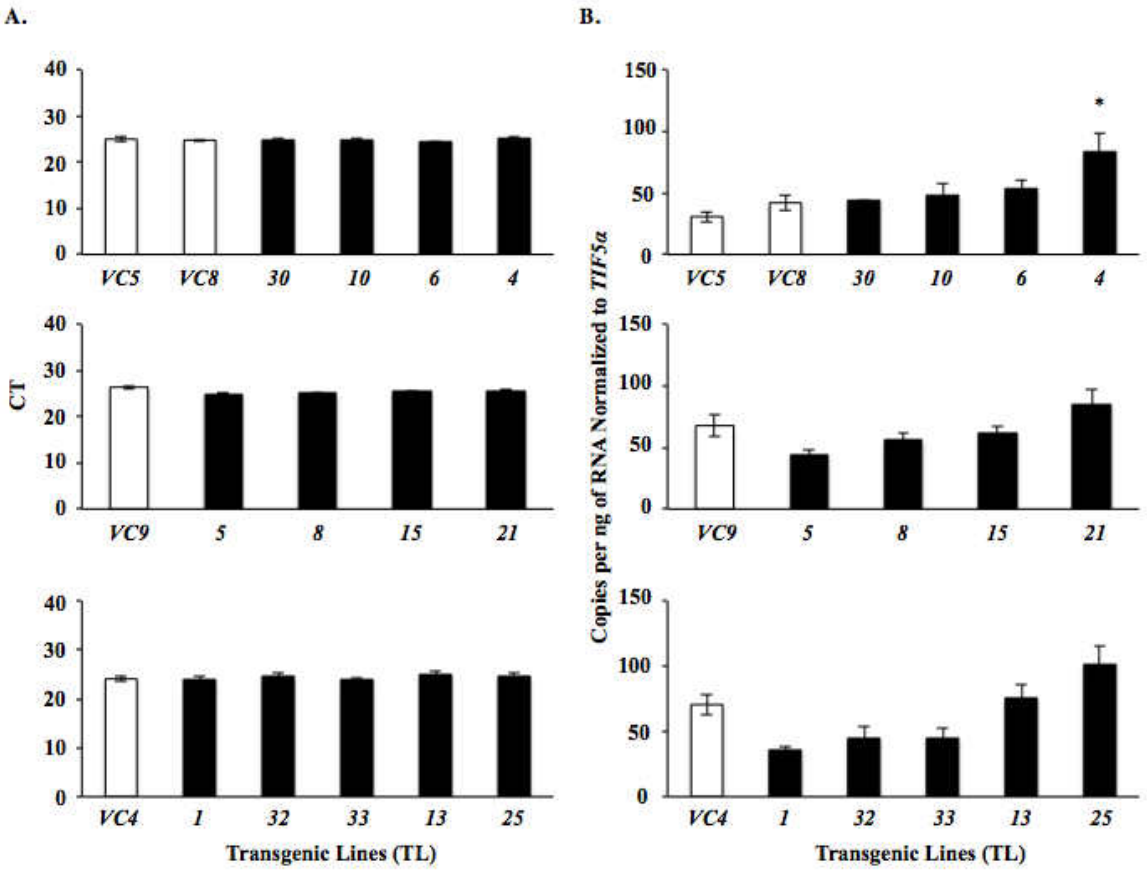

Figure 36: qPCR expression analysis of *10s12800* in *Pt x Pa* transgenic lines. A) *TIF5α* cycle threshold values for thirteen transgenic lines (TL, indicated in black) and 4 vector controls (VC, indicated in white). B) Copies per ng of RNA normalized to translation initiation 5 alpha (*TIF5α*) expression of *10s12800* normalized to *TIF5α*. Only *TL4* shows a statistically significant two-fold increase in expression over the *VC8* vector control. Bars indicate mean +/- SE.

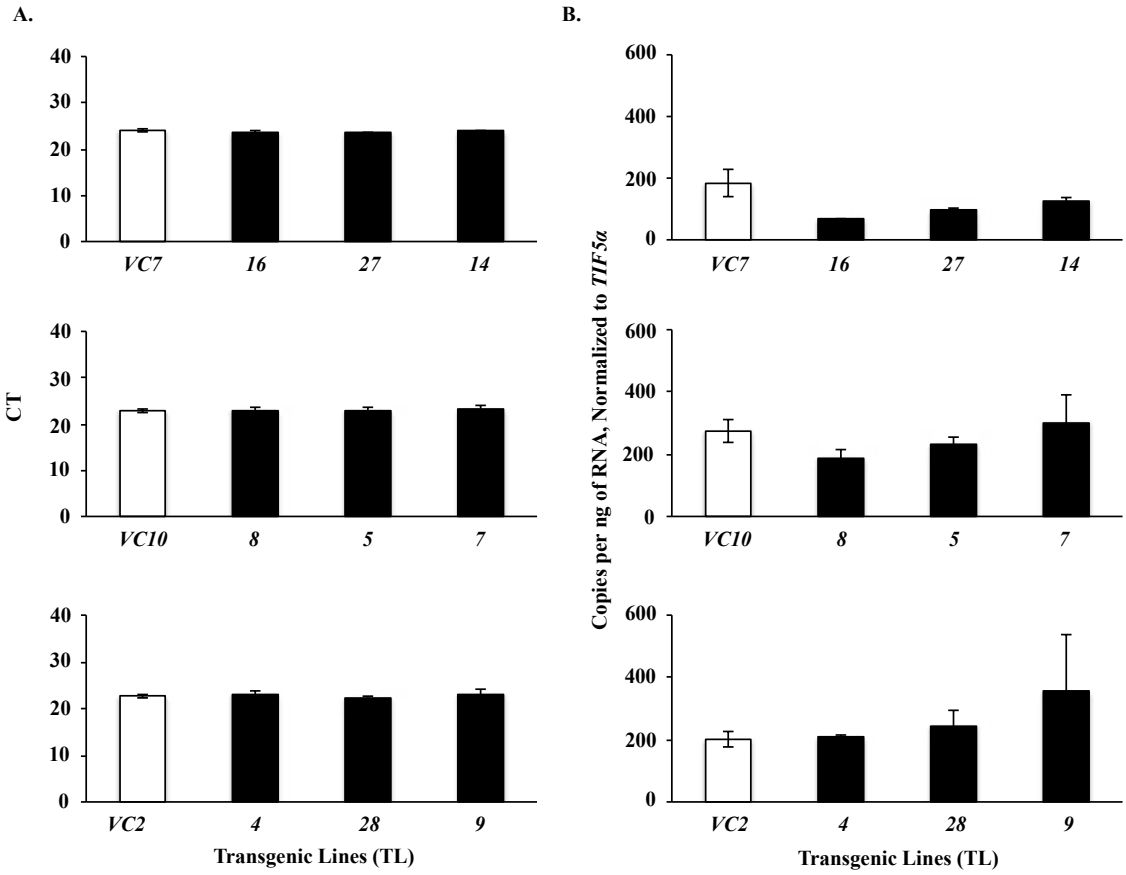

Figure 37: qPCR expression analysis of *10s12800* in *Pc x Pg* transgenic lines. Left had side shows CT values of *TIF5α* for nine transgenic lines (TL, indicated in black) and 3 vector control lines (VC, indicated in white). Right side shows absolute expression of *10s12800* relative to *TIF5α* for each transgenic and VC. Bars indicate mean +/- SE. N= five biological replicates.

expression between TL and VC lines will not result in a detectable difference in choice bioassays. Because *TL4* was the only transgenic line to show an effect, albeit small, we conducted a single on-tree no-choice bioassay to determine if the mutant line had an affect on WMTM weight gain. Twelve late 2nd instar WMTM larvae were caged with meshbags on nine *TL4* and *VC8* trees for seven days. We did not observe a difference in fresh weight gain for insect (figure 39B; t-test, $p>0.05$, $n=9$). The small 2 fold increase

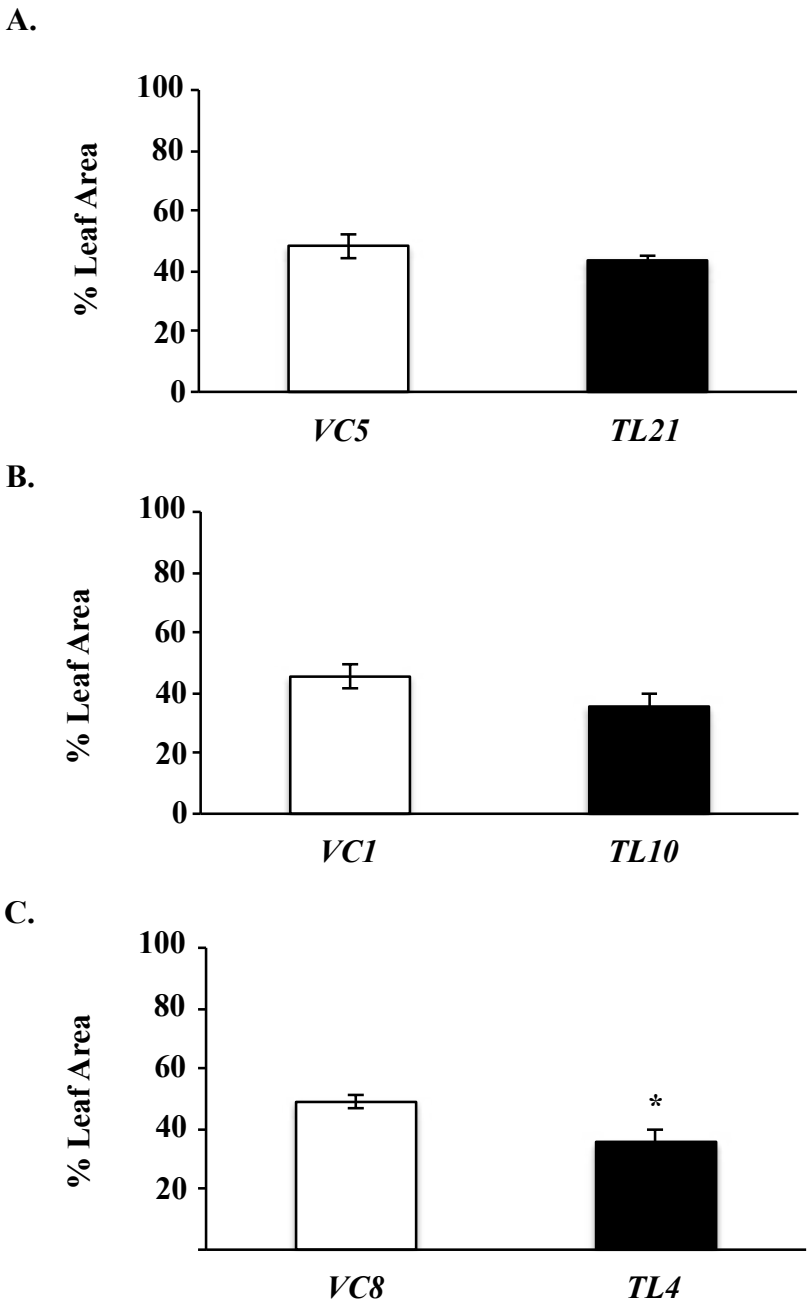

Figure 38: Choice Bioassays of Select Transgenic Lines in *Pt x Pa* Background. Three choice bioassays comparing feeding preference of a *10s12800* transgene line versus a vector control were conducted. A) WMTM did not prefer to feed on *TL21* transgene line versus vector control (Nested ANOVA, $p>0.05$, n=8 pairs). In a choice assay between *TL1* vector and *TL10* transgene lines WMTM consumed more leaf material from from the control but was not found to be statistically different (Nested ANOVA, $p>0.05$, n=8 pairs). C) WMTM consumed more leaf area (27% more) from the *TL8* control line compared to the *TL4* transgenic line (Nested ANOVA, $p<0.05$, n=8 pairs). All bars are mean +/- SE.

A.

B.

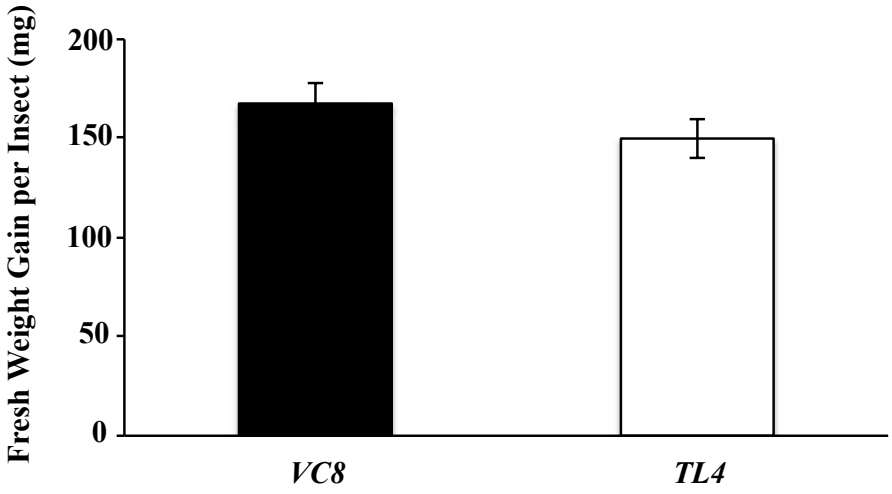

Figure 39: On-Tree No-Choice Development Bioassay Comparing Weight Gain of WMTM Caged on *VC8* or *TL4*. A) Image of mesh bags on trees for on-tree no-choice bioassay. B) WMTM did not gain more fresh weight when reared on *TL4* compared to *VC8* trees (t-test, $p<0.05$, n=9 trees per genotype).

in *10s12800* expression fold increase had no detectable effect on growth of WMTM larvae. Insects appear to have gained, on average, more weight on both the *TL4* and *VL8*

than the previous on-tree experiments, suggesting insects were healthy and able to consume the leaves. The bioassays suggest that there may be a slight feeding preference for *VC8* over *TL4* in choice bioassays but that preference does not appear to translate to an effect on insect weight. It is possible that because the difference in *10s12800* gene expression is smaller it may not have an impact on insects that are restricted to one leaf source.

Discussion

In terms of full disclosure, our initial attempts to identify T-DNA insertions involved other PCR methods that required additional enzymatic DNA manipulation and and cloning steps and these approaches were not successful. Ultimately we switched to TAIL and SiteFinding PCR instead as they have been successfully applied in *Populus* and *Arabidopsis* and were considerably easier to use and achieve consistent results between members of the Ralph lab. Both strategies were used not only to identify possible T-DNA insertions within *E8-16*, but also to independently confirm results between PCR strategies. In addition, we used multiple degenerate primers to ensure we could find a few that would work. For SiteFinding PCR we used two different degenerate primers and for TAIL PCR we used seven different degenerate primers. SiteFinder-1 and 2, as well as products from TAIL (AD 22, AD3, and AD4) reactions resulted in sequences that contained a perfect match to part of the T-DNA as well as the same single location within chromosome 10. This was significant as the amount of replication within and between methods was extensive. As mentioned in the introduction, studies by Busov et al (2010) and Harrison et al. (2007) showed that while most AT mutants contain a single T-DNA, other mutants had additional insertions. We would expect that at this level

of replication using two different PCR methods we would have identified multiple T-DNAs within the mutant if they existed. Our mutant *E8-16* probably contains a single T-DNA insertion and a southern blot would provide confirmation.

Once we identified a T-DNA site we had to determine if nearby genes were activated. In our qPCR analysis of genes near the AT insertion site two genes, *10s12800* and *10s12790*, revealed a higher expression in the *E8-16* mutant versus *Pt x Pa* trees. While *10s12790* was over-expressed in the mutant it was a small increase in expression (1.53 fold). The microarray, which will be discussed a little later, did not indicate this gene as over-expressed. Limited evidence from the qPCR analysis suggests that at best this gene is only slightly impacted by the enhancers and is unlikely to be causing the IR phenotype. On the other hand, *10s12800*, the gene closest to the T-DNA site had a nearly 7 fold increase in expression with strong statistical support. In addition the gene was one of the most strongly over-expressed in the microarray suggesting that it is strongly impacted by the T-DNA enhancers. Therefore, we believe we had the desired outcome of a single vector causing transcriptional activation of a single gene (*10s12800*) within *E8-16*.

10s12800 is a Putative E3 RING-H2 Ubiquitin Ligase

Based on BLAST results and CLUSTAL alignment of proteins with similar amino acid sequences from the plant kingdom it appears that *10s12800* is an E3 RING-H2 ubiquitin ligase. E3 ligases function as protein regulators by tagging proteins with ubiquitin for degradation within the 26S proteasome/ubiquitination pathway. Ubiquitination is a multistep reaction involving three enzymes: ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2), and ubiquitin ligase (E3) (figure 40;

Glickman and Ciechanover 2002). Within *Arabidopsis* more than 5% of all genes are predicted to be involved in the 26S proteasome/ubiquitination pathway, suggesting that protein ubiquitination is essential in a number of biological processes for plants (Smalle and Vierstra 2004). Over 1300 E3 ubiquitin ligases have been predicted in *Arabidopsis* compared to 2 isoforms of E1 and 37 predicted E2s, suggesting that E3 ligases have a role in substrate specificity in ubiquitination (Smalle and Vierstra 2004). Little work has

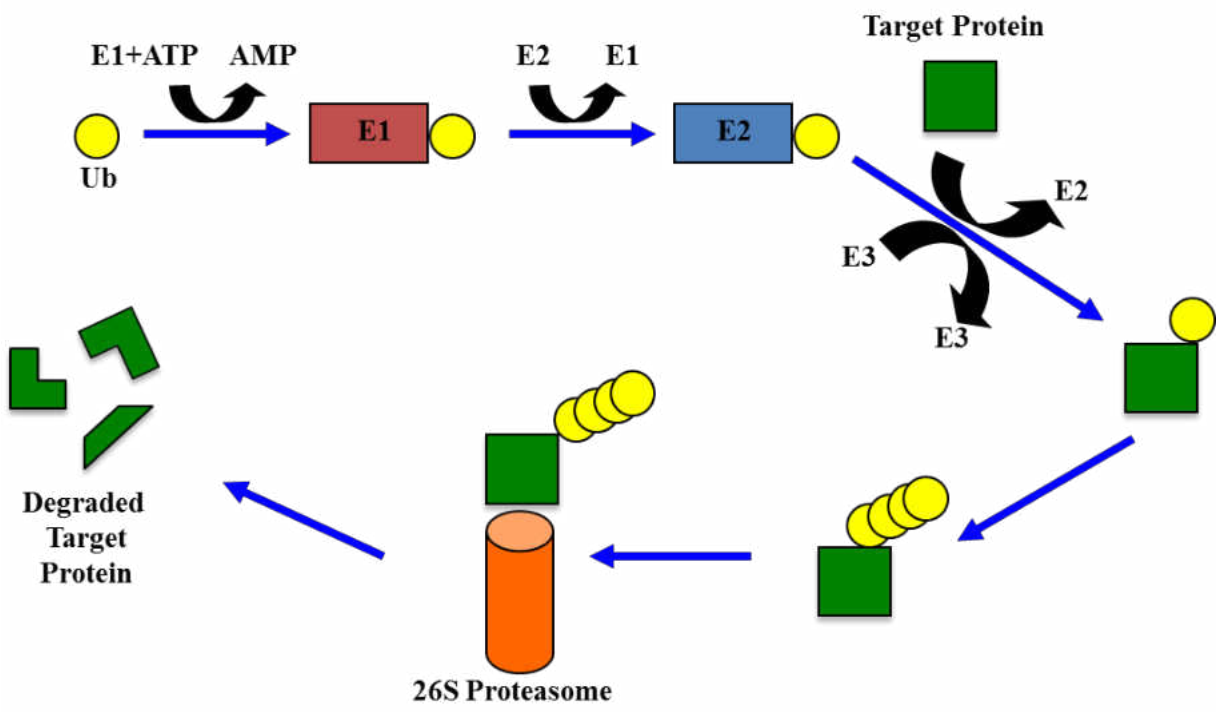

Figure 40: 26S proteasome/ubiquitination pathway. Ubiquitin is attached to ubiquitin activating enzyme (E1) with adenosine triphosphate (ATP) and then transferred to ubiquitin conjugating enzyme (E2). Ubiquitin ligase (E3) helps transfer Ub to a target protein. Once the target is tagged with one or more Ub the 26S proteasome recognizes the protein and degrades it.

been done to identify all of the E3 ligases in *Populus* but there are probably at least 1300 and possibly more due to genome duplication.

E3 ligases can be further divided into three classes based on domain sequences: HECT, U-box, or RING (Smalle and Vierstra 2004). Based on our bioinformatics

analysis, we believe 10s12800 is an E3 RING ubiquitin ligase. RING E3 ligase domains are 40-60 amino acids long containing eight Cys and/or His residues that coordinate with zinc ions in a cross-brace structure (Barlow et al. 1994; Borden et al. 1995; Borden 2000) for protein-protein interactions (Lovering et al. 1993; Borden 2000). The domain is thought to function as a scaffold to bring the E2 with the thioester-linked ubiquitin and a specific protein substrate together for ubiquitination (Lorick et al. 1999).

RING domain-containing proteins can be further divided based on other conserved domain features as well as their ability to function as either simple or complex E3 ligases (Freemont et al., 1991; Freemont 1993). RING domains can be categorized as based on the composition of Cys and His residues. The two main types, C3HC4 (RING-HC) and C3H2C3 (RING-H2) are both important for enzyme activity in E3 ligases (Lorick et al. 1999) but differ at metal ligand position 5 (Freemont 1993; Lovering et al. 1993). Because our E3 ligase protein, 10s12800, contains a histidine residue at ligand position 5 it has been classified as a RING-H2 type protein. The other conserved domain, a predicted transmembrane domain at the N-terminal of 10s12800, suggests that the protein is in a membrane. To date we do not know if it is localized within the plasma membrane or an organelle membrane or both. RING ligases can also be classified as simple or complex based on interactions with proteins. Simple RING proteins can contain a substrate-binding domain and E2-binding RING domain within the same protein or act as homodimer/heterodimer complex with another RING protein. Other RING ligases are classified as complex and are often referred to as CUL1-Based or SCF (SKIP1-like Cul1 F-box) complexes in that they have cullin protein that recruits a RING-H2 at its C-terminus while the N-terminal binds to substrate adapter proteins (Kuroda et al. 2002;

Lechner et al. 2002; Risseeuw et al. 2003). Ultimately our knowledge about 10s12800 is based on the amino acid sequence and identification of conserved domains. We feel confident that the predicted function based on homology is correct since all of the sequence hallmarks for an E3 RING-H2 ligase are present in the correct positions in 10s12800. Nonetheless, bench experiments are needed to confirm gene function. Future experiments should assess enzyme activity and identify protein-protein interactions to confirm class/type of protein (chapter 5).

RING proteins have been associated with plant growth and development as well as environmental interactions (Schweichheimer et al. 2009). A literature review on E3 ligases and their impact on regulatory pathways can be found in Chen and Hellmann (2012). The authors compiled a list of E3 ligases with known classes and their involvement in plant processes. Monomeric RING and CUL proteins have been shown to be involved in a wide variety of pathways including: stress related responses such as water, salt, and drought (Cheng et al. 2012; Ning et al. 2011; Lee et al. 2009; Seo et al. 2012); plant hormone signaling involving ethylene (Lyzenga et al. 2012; Guo and Ecker 2003; Potuschak et al. 2003; Gagne et al. 2004; Binder et al. 2007, An et al. 2010; Yoshida et al. 2005; Christians et al. 2009, Thomann et al. 2009), auxin (Dharmasiri et al. 2005; Kepinski and Leyser 2005; Tan et al. 2007; Pan et al. 2009; Calderon Villalobos et al. 2012), jasmonic acid (Devoto et al. 2002; Chini et al. 2007; Thines et al. 2007; Yan et al. 2009; Sheard et al. 2010; Wager and Browse 2012), gibberellin (McCinnis et al. 2003; Dill et al. 2004; Ariizumi and Steber 2007; Ariizumi et al. 2011); as well as plant morphological characteristics like nodule organogenesis (Yuan et al. 2012), root gravitropism and growth (Sakai et al. 2012), shoot and root development (Stirnberg et al.

2007; Mashiguchi et al. 2009; Puig et al. 2012), and flowering (Pazhouhandeh et al. 2011). RING E3 ligases are continually being discovered in forward genetic screens (Bueso et al. 2014; Huang et al. 2010). It therefore comes as no surprise that we have identified an E3 RING-H2 ligase, that when over-expressed, appears to be involved in plant defense against insect herbivory.

Global Expression Analysis

The biggest potential clues about how over-expression of *10s12800* is causing IR in the *E8-16* mutant come from the Agilent 4x44 microarray study. Surprisingly, our microarray experiment revealed only a small number (22) of genes as differentially expressed between *E8-16* and *Pt x Pa* leaves. Quality control procedures indicated that the RNA was of high integrity and the hybridization signals were consistently strong across microarrays. Genes tested in the microarray validation showed similar expression effects and therefore we feel confident that our microarray is providing an accurate representation of differentially expressed genes.

Of particular interest is the over-expressed MADS-box and class 1 chitinase families. MADS-box genes are transcription factors that have diverse biological functions. These genes involved in many early developmental processes including phase transitions in sporophytic development; flower and fruit development; and root and leaf morphogenesis (Smaczniak et al. 2012). These proteins are also widely conserved in a variety of organisms, including bacteria, fungi, and animals. However, the gene family is significantly larger in higher plant species suggesting duplication events have allowed for divergence in function within the family (De Bodt et al. 2005). Apart from a few studies exploring MADS-box transcription factors and their role against pathogens, very little

work has been done in any plant species to explore their role in plant defense. Down-regulation of the MADS-box transcription factor *OsMADS26* in *Oryza sativa* resulted in pathogen resistance to rice pathogens *Magnaporthe oryzae* and *Xanthomonas oryzae* (Khong et al. 2015). Zhang et al. (2016) determined that transgenic lines silencing the MADS-box transcription factor *NbMADS1* from *Nicotiana benthamiana* inhibited stomatal closure, decreased hydrogen peroxide, and reduced hairpinXoo-induced resistance to *Phytophthora nicotianae* compared to control plants. Application of hydrogen peroxide and sodium notroprusside resulted in a return to the normal stomatal closure phenotype observed in control plants suggesting that the MADS-box is important in responding to hairpinXoo. To date there is a lack of literature looking at *Populus* or other plant MADS-box transcription factors and their possible role in IR. Future work that could be done to explore these MADS-box transcription factors and their possible role in IR are discussed in chapter five.

Chitinases are glycosyl hydrolases that can hydrolyze the glycosidic bonds between two or more carbohydrates. Chitinases are present in a wide variety of yeasts, plants, actinomycetes, arthropods, and humans (Hamid et al. 2013). In plants, chitinases function in the defense against insects by destroying chitin, an important scaffold material supporting the cuticles of the epidermis and trachea, as well as the peritrophic membrane of the gut epithelium (Hamid et al. 2013). Lawrence and Novak (2006) showed that a *Populus* chitinase, inserted within tomato, resulted in retardation of development of the Colorado potato beetle. We suspect that one or more members of the class 1 chitinases are causing the IR phenotype in *E8-16* and future work could include creating transgenics and studying gene function within this group of proteins. We

hypothesize over-expression of *10s12800* results in tagging a negative regulator of class 1 chitinases for protein degradation by the ubiquitination/26S proteasome pathway. Without the negative regulator MADS-box transcription factors can then allow transcription of class 1 chitinases (figure 41). Future work that could be done to confirm this hypothesis (chapter 5).

Transgenic Recapitulation of the Phenotype

One of the most important steps in conclusively determining whether or not a gene is involved in the phenotype from AT mutants is to recapitulate the phenotype in one genetic background. In the *E8-16* mutant, we observed a seven fold increase in *10s12800* expression. Therefore, our goal was to create new transgenics that over-expressed *10s12800* at a level similar to the original mutant (i.e., 5-10 fold) and then conduct bioassays to determine if the same IR phenotype occurs. Our collaborators at NDSU were able to generate 12 transgenic lines within the *Pt x Pa* background and 9 lines within the *Pc x Pg* background. Unfortunately we were unable to achieve the desired fold change between TL and VC lines. One possible reason for our limited success is that the pCAMBIA S1300 vector used may not have been the best choice for our particular gene. The type of promoter within the vector can effect the magnitude of transgene expression in transformants (De Bolle et al. 2003). For example, a common vector used to over-express genes in transgenics is the 35S promoter of the CAMV. DeBolle et al. (2003) found a bimodal distribution with few transgenic lines having high expression levels and most transformants having low expression. Other promoters, such as the mannopine synthase gene, never reach the high expression levels of those with the 35s promoter but have more normal distributed of expression levels in transformant

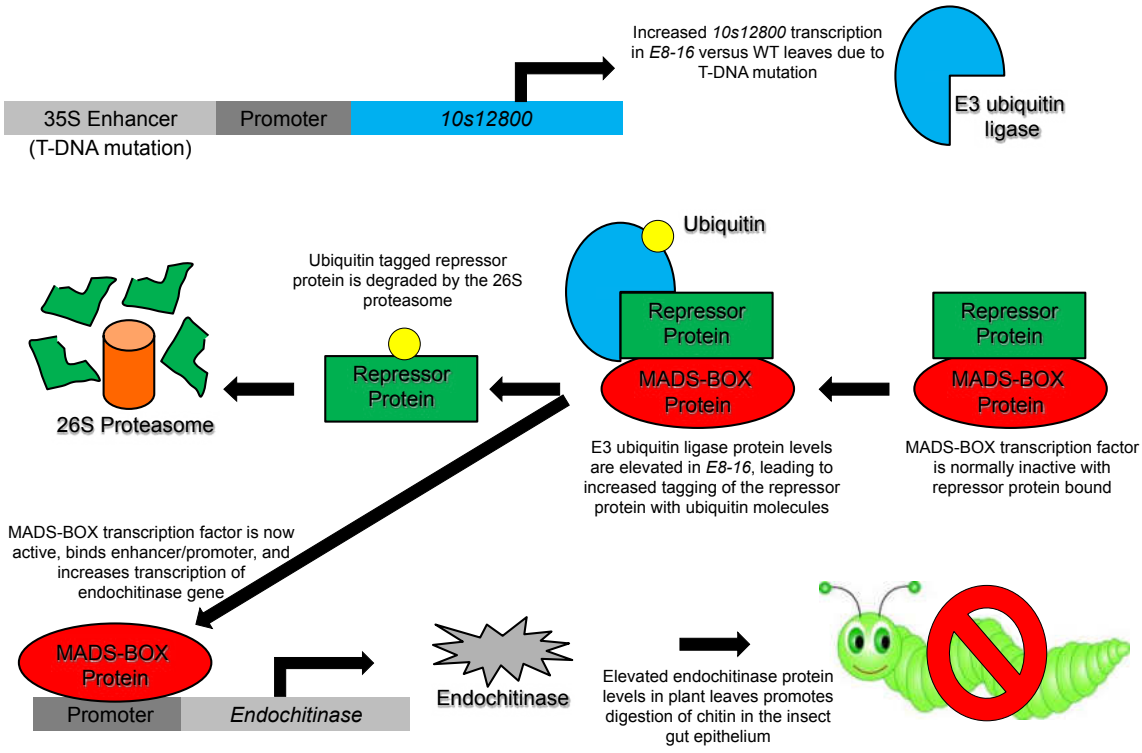

Figure 41: Hypothesized mechanism of action for *10s12800*, leading to increased insect resistance. E3 ubiquitin ligases target specific proteins for degradation by adding ubiquitin peptide tags, thus marking the protein for digestion by the 26S proteasome[6]. We propose that the 10s12800 protein targets a negative regulator of plant defense that, presumably, controls the activity of one or more transcription factors involved in activating defense gene expression in response to insect attack. Possible transcription factor targets from the microarray data include the MADS-BOX, bZIP and No Apical Meristem (NAM) families. Given the established role of endochitinases in plant defense, we propose that the transcription factor, once released from negative inhibition, activates expression of an endochitinase, which then acts to destroy chitin in the peritrophic membrane of the insect pest gut epithelium that ultimately deters insect feeding and disrupts growth and development.

populations (De Bolle et al. 2003). Our vector has a superpromoter that contains the mannopine synthase gene. Perhaps the vector is incapable of delivering the large increase needed to recapitulate expression in the *E8-16* mutant. It is also possible that other factors may be influencing *10s12800* expression. Transgene expression can be influenced by surrounding regulatory sequences or incorporation into chromosomal regions with higher or lower transcriptional activity (Meyer 2000). It is also possible that transcriptional gene

silencing is occurring due to methylation of promoter sequences of the transgene (Mette et al. 2000). We did observe a few lines where the overall expression of *10s12800* was lower in transgene lines than vector controls. qPCR screening for *10s12800* expression for all transgenic lines only produced one line, *TL4*, with a small two-fold increase in expression that was statistically higher than the VC. This mutant did show a small negative effect on WMTM feeding preference in the choice assay.

In an on-tree no-choice assay the *TL4* mutant did not reveal a statistical difference in insect weight gain. While it was not statistically different the weight gain for insects feeding on *TL4* was slightly smaller. We believe that because the two-fold expression increase is a very small difference in expression it had a smaller effect on the insects than the original mutant. The data is not conclusive but does suggest additional work can be done to strengthen the case that *10s12800* is causing the IR phenotype in *E8-16*. Additional on-tree no-choice development bioassay experiments with greater biological replication may reveal a small statistical difference in feeding preference and insect weight growth with the current *TL4* mutant. We could also use petri plate no-choice bioassays to determine if *TL4* has an effect on insect weight gain and development. In chapter 3 we conducted two separate experiments for all three bioassays to ensure *E8-16* was an IR mutant. We only conducted one choice and one on-tree no-choice assay for *TL4*. Variables such as tree or insect health and insect behavior could be playing a role in our observed results. However, it should be noted that insect and tree health appeared to be good before and during the bioassay and insect weight gains was substantial on both the *VC* and *TL4* trees. Another member of the Ralph lab is creating additional transgenic mutants to produce lines with a higher fold increase in *10s12800* expression for eventual

bioassay confirmation. In terms of objective #3 we have identified the *10s12800* gene as a putative E3 RING-H2 ubiquitin ligase, determined it is expressed in all tissues but most strongly in mature leaf tissues, and the microarray revealed a few different classes of proteins that appear to be impacted from the mutation. We hypothesize that over-expression of certain class 1 chitinases may be causing IR within the *E8-16* mutant.

Conclusions

Molecular work conducted on the *E8-16* genome revealed a T-DNA insertion at chromosome 10 that appears to be causing over-expression of the gene *10s12800*, an E3 RING-H2 ubiquitin ligase. Characterization of the mutant further further revealed that the gene is ubiquitously expressed in *Populus* tissues and appears to cause over-expression of a limited number of genes including MADS-Box transcription factors and chitinases. We hypothesize that over-expression of 10s12800 causes over-expression of either MADS-box or bZIP transcription factor that ultimately leads to over-expression of one or more class 1 chitinases. Further work needs to be completed to not only fully characterize *10s12800* but also to potentially link a gene pathway that explains how *E8-16* is IR to WMTM. While we have begun to characterize this E3 ligase there are still many questions that could be answered with further work. These possible future directions will be covered in chapter 5.

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CHAPTER V

EPILOGUE

Introduction

The overall goal of this Ph.D. dissertation was to conduct a forward genetics screen of AT *Populus* mutants to identify genes involved in IR for possible incorporation into breeding programs to develop superior trees for plantation forestry. In total, over 7000 choice assay plates were prepped, scanned and analyzed over the course of two and half years to screen 770 AT *Populus* mutants from the MTU population and evaluate candidates identified from a previous screen of the QU population (Ralph 2009). From the forward genetic screen we showed we could use unwounded, wound local, and wound systemic choice bioassays in mutant-mutant comparisons. The initial screen was followed by mutant versus wildtype choice bioassays to identify candidates with altered constitutive and induced IR. In addition, we also investigated candidates identified from a prior screen for IR (Ralph 2009). WMTM preferred to consume unwounded leaves from *Pt x Pa* wildtype trees over the *E8-16* mutant in two independent unwounded choice bioassays. Because a reduced feeding preference does not necessarily mean the mutant impacts insect development, we also conducted no-choice bioassays to compareinsect growth and development. Results confirmed that *E8-16* had a negative impact on WMTM larvae by delaying their development and negatively impacting weight gain.

Because *E8-16* showed consistent results as an IR mutant we continued with molecular work to achieve objective #2 of the dissertation. SiteFinding and TAIL PCR identified a T-DNA on chromosome 10. The "activated" gene, *10s12800*, was identified using qPCR and had the expected 5-10 fold increase in expression typical of AT mutagenesis. A bioinformatics analysis of the10s12800 amino sequence revealed the gene to be a putative E3 RING-H2 ubiquitin ligase that is likely involved in the 26S proteasome/ubiquitination pathway.

To characterize *10s12800* (objective #3), we examined constitutive tissue expression in *Populus*, as well as a microarray to exam global transcriptome differences between *E8-16* and *Pt x Pa* to identify gene regulatory networks influenced by the mutation. We also attempted to develop new transgenic lines over-expressing *10s12800* to recapitulate the phenotype from the original AT mutant. qPCR analysis of *10s12800* in *Populus* tissues revealed that this gene is expressed in all tissues, with highest expression in mature leaves and petioles. The microarray analysis revealed a small set of genes that are over-expressed in the *E8-16* mutant, including *10s12800*, transcription factors (MADS-Box, NAM, bZIP), and chitinases. Transgenic lines created to over-express *10s12800* revealed one line, *TL4*, with a two-fold increase in expression. Bioassay results revealed a feeding preference of WMTM for vector controls over *TL4* but we did not detect a difference in insect growth and development from the on-tree no-choice assay.

There are several possible future directions that could be pursued based on our findings. The following sections reveal future directions for: 1) AT mutant candidates discovered from the forward genetic screen, 2) the *10s12800* gene in regards to its

function and role in plant defense, and 3) experiments that could be conducted on genes identified from the microarray to be connected to *10s12800*.

Future Directions

Candidates from the Forward Genetic Screen

Forward genetic screening in trees is a time consuming process. The propagation and growing of healthy *Populus*, sometimes up to four months for a single round of bioassays, is physically demanding and time consuming. Greenhouse space, environmental factors such as natural sunlight during certain times of the year, tree health, and availability of healthy WMTM insects, can greatly impact the speed at which trees can be screened for altered IR. As discussed in chapter 3, replication is also required to ensure repeatability of experiments with different insect batches. While it would have been desirable to explore additional candidates identified from the MTU screen more thoroughly, we ultimately decided to focus our time, energy, and funding towards investigating *E8-16*.

Future directions for research might involve screening new AT mutants for altered IR or conduct additional bioassays with greater biological replication on candidates identified from the MTU screen. Several candidates from the MTU screen have shown substantial differences in feeding preference between mutant-mutant and mutant-wildtype rounds of the screen for one or more choice bioassays. While some will turn out to be false positives we would expect that some have altered IR. These mutants would then undergo molecular work including T-DNA mapping and identification of the "activated" gene(s). This dissertation contains protocols for conducting choice and no-choice bioassays, as well as the molecular procedures for T-DNA mapping and

identifying the activated gene(s). New members of the Ralph labwill be able to more rapidly jump into research to find other potential mutant candidates and move into new and different experiments to characterize genes of interest.

10s12800 Gene

Generation of additional transgenic lines over-expressing *10s12800*.

Unfortunately we were unable to generate transgenic lines recapitulating the same fold change of *10s12800* expression observed in the original *E8-16* mutant. Creating *Populus* transgenic lines is a time consuming process requiring as much as a year of work from the early stages of *Agrobacterium* transformation and plant tissue culture to young trees growing in the greenhouse ready to be screened for gene expression. As such, by the time we received transgenic lines from NDSU and had completed the qPCR screening of the transgenic lines, we were running short on time to generate additional mutants under the NSF grant. As discussed in chapter 4, confirmation that a gene is responsible for the observed phenotype in AT only requires recapitulation of the phenotype in one transgenic line. We tested 22 transgenic lines but only had one line with a small 2 fold change in *10s12800* expression that showed a negative impact on WMTM feeding preference in choice assays but not a statistical effect in weight gain in on-tree no-choice assays.

As described in chapter four, we are uncertain as to why we were unable to achieve the transgenics with the desired level of *10s12800* expression. We are not sure if the problem was due to the promoter (De Bolle et al. 2003), unfortunate luck that transgenic expression was inhibited by surrounding regulatory sequences, or incorporation of the transgene into chromosomal regions with higher or lower transcriptional activity for many of the lines (Meyer 2000), or epigenetic silencing

(Huettel et al. 2006), or a combination of these factors. One possible course of action may be to continue creating additional transgenic lines with pCAMBIA S1300 and screening them for *10s12800* expression using qPCR. If the problem is due to the promoter or where the transgene is inserting within the *Populus* genome then with enough screening we could potentially find a few transgenics with the desired *10s12800* expression. The alternative is to attempt new transgenics with a different promoter. Other plasmids with varying numbers of CAMV virus promoter sequences have been used to over-express genes within plant species (Park et al. 2005; Gaxiola et al. 2001; Harrison et al. 2007, Plett et al. 2010). A member of the Ralph lab is currently working towards creating new transgenics over-expressing *10s12800*. Once trees are created bioassays can be conducted to validate the IR phenotype.

Ubiquitination assay. While the amino acid sequence suggests that *10s12800* may be an E3 RING-H2 ubiquitin ligase, future work could be done to confirm gene function by testing the gene's enzyme activity. Many E3 ligases have the ability to regulate their own activity by auto-ubiquitination *in-vitro*. Ubiquitination reactions could be performed by using an auto-ubiquitination kit (Enzo Life Sciences) and then separated using SDS-PAGE. Immunoblot analysis could then be conducted by performing a western blot using the kit's anti-ubiquitin antibody (Ab) to detect auto-ubiquitination and anti-GST Ab (Santa Cruz Biotech) to detect Ub moiety. Fortunately, one member of the Ralph lab is currently working on this experiment, which would go a long ways towards characterizing this gene.

Yeast two-hybrid. E3 ligases function within the 26S proteasome/ubiquitination pathway by tagging proteins with ubiquitin for degradation. Proteins that interact with

10s12800 and are subsequently degraded could cause an increase in expression of defensive proteins in the *E8-16* mutant, leading to IR. Therefore, we would ultimately like to know which *Populus* proteins might be interacting with 10s12800. One method designed to determine protein-protein interactions is called a yeast two-hybrid. The classic yeast two-hybrid system created by Fields and Song (1989) was designed so that protein interactions could be detected by the activation of a reporter gene. A bait protein, typically the known protein of interest, is expressed as a fusion protein to a DNA binding domain of a transcription factor, such as GAL4 or LexA. The DNA binding domain of the fusion protein can then bind to the operator sequence in the promoter region of a reporter gene. However, without the remaining portion of the transcription factor, called the activation domain, no transcription activation of the reporter gene occurs. If other proteins fused with the activation domain, called prey proteins, interact with the bait protein they will fully reconstitute the transcription factor and activate the reporter gene.

Yeast two-hybrid experiments are typically not performed by testing a single prey protein. Instead a larger collection of unknown preys expressed from cDNA or genomic libraries are screened for potential interactions to the known bait protein(s). Large-scale screening can be done using two approaches: library screening or a matrix (Auerbach and Stagljar 2005). In the library screening approach a liquid culture of bait fusion proteins in one yeast reporter strain is mixed with a library pool of preys in another yeast strain and incubated and plated on selective media. Those with protein interactions between the bait and prey are selected for plasmid isolation and sequenced, and a BLAST analysis can be done to identify the prey protein. In the matrix approach a defined collection of preys are used by spotting their yeast clones onto plates. The bait protein is then mated to the prey

proteins and then transferred to a selective media. Unlike the library method, prey proteins are already known so further steps to isolate and sequence plasmids are not required (Auerbach and Stagljar 2005)

Until now we have mostly described the classic yeast two-hybrid method in very broad terms of how it works and its application. In terms of using this approach to finding proteins interacting with 10s12800, one very significant limitation exists. In the classic yeast two-hybrid assay, protein system interactions must occur in the nucleus for the reporter gene to be activated. This presents a potential problem. Based on the bioinformatics analysis in chapter 4, 10s12800 appears to contain transmembrane domains suggesting it is a membrane bound protein. While we could attempt to use the classical method by truncating the 10s12800 protein it would likely lead to altered protein folding, resulting in false positive protein interactions or no detectable protein-protein interactions.

Fortunately, modifications of the initial yeast two-hybrid system now exist to identify a greater variety of protein interactions including protein-protein interactions between non-soluble membrane proteins. The split ubiquitin yeast two-hybrid system allows for the study of protein-protein interactions for proteins found outside the nucleus (Johnsson and Varshavsky 1994). In the future a split ubiquitin yeast two-hybrid system may lead to the discovery of a negative regulator of genes involved in IR.

Impacts of environment on phenotype. As described in Busov et al. (2010), more AT *Populus* trees showed a visible phenotype after multiple years in the field (6.5%) than found in greenhouse (1.6%). All of our experiments with *E8-16* were done in a greenhouse environment. While we did not observe any noticeable phenotypic

differences from *E8-16* in the greenhouse, perhaps transgenic lines over-expressing *10s12800* in a long-term field trial may reveal a visual phenotype in response to environmental stress. To conduct this type of experiment would: 1) require permits to plant AT trees within the field and, 2) require using a *Populus* background that is hardy enough to survive North Dakota winters. The *Pc x Pg* genetic background that we tried to use to recapitulate the phenotype in chapter 4 is a hardier hybrid designed to survive Minnesota winters. We could attempt to create additional transgenic lines within this genetic background and, providing permit approval, conduct a long-term growth study to focus on environment affects on phenotype in trees over-expressing *10s12800*.

In addition to conducting a long-term field study, new experiments could be done testing *10s12800* transgenic lines within the greenhouse to other biological stresses, such as additional insect species and pathogens. In our work with *E8-16* we only tested effects on consumption, growth, and development of WMTM. As discussed in chapter 2, hundreds of insect species feed on *Populus*. To date we have not tested *E8-16* to determine if it has a negative impact on other lepidopteran species, such as the large aspen tortrix (*Choristoneura conflictana* Walker), common aspen leaf miner (*Phyllocnistis populiella* Chambers) or gypsy moth (*Lymantria dispar* Linnaeus). Just because *E8-16* had an impact on WMTM does not mean it will negatively affect other lepidopteran species. In the case of the invasive gypsy moths we would have to perform bioassays in a facility that has the appropriate permits to house this species in the U.S. We have not tested *E8-16* against non-lepidopteran species that feed on leaves or other *Populus* tissues. *10s12800*, while expressed lower in tissues other than leaves and

petioles, could have an impact on phloem sucking and wood boring insects. New bioassays and experiments could be designed to explore these plant-insect interactions.

Future Targets

From the Agilent 4x44K microarray study we identified several genes that were over-expressed within *E8-16* leaves that could be potential targets for future experiments. MADS-box transcription factors from *Populus* have been analyzed for their role during flower development and vegetative development (Zhang B et al. 2008; An et al. 2011; Ceske et al. 2003; Ceske et al. 2005; Cseke et al. 2007). Apart from a few studies exploring MADS-box transcription factors and their role in defense against pathogens (Khong et al. 2015; Zhang et al. 2016) very little work has been done in any plant species to explore their role in plant defense.

As discussed in the beginning of chapter 3, over-expression in a microarray does not necessarily mean those genes cause IR. One possible future experiment could be to create transgenic *Populus* over-expressing and knocking out expression of MADS-box transcription factors identified from the microarray. Following the production of transgenic lines and verification of expression of the intended genes using qPCR, choice and no-choice assays could be done using WMTM to determine if they play a role in IR. In-depth phenotyping following a growth trial of transgenic trees could also be done to see if these MADS-box transcription factors alter growth or result in a visible phenotype in either vegetative or floral tissues.

Some of the other gene families identified in the microarray, such as bZIP transcription factors, NAM proteins transcription, and chitinases, are regularly found to be over-expressed in transcriptomic studies following actual or simulated herbivory in

Populus (Ralph et al. 2008; Major and Constabel 2006; Christopher et al. 2004). Creation of transgenics over-expressing these genes within a *Populus* background, followed by bioassays would be a logical step to determine if they cause IR. Chitinases are a large gene family with amino acid similarity. Transgenics could be particularly useful in separating out chitinases that are relevant to IR identified from the microarray.

Ultimately we would want to identify how the insect is being impacted from the *E8-16* mutant. Future experiments could be done to determine if certain chitinases from the microarray actually cause physical damage to the peritrophic membrane in WMTM. Kabir et al. (2006) examined the use of a chitinase from *Bombyx mori* as biocontrol agent for Japanese pine sawyer (*Monochamus alternatus*). Starved insects were gavaged with purified chitinase or Na-phosphate buffer and then then allowed to feed on pine branches for two days. Insects were dissected and the midgut was separated from the rest of the digestive tract and then cut into three sections and excised to expose the peritrophic membrane. Peritrophic membranes were prepared for fluorescence microscopy using chitin binding fluorescence dye or were prepared for scanning electron microscopy. The fluorescence assays and scanning electron microscopy revealed that the peritrophic membrane was damaged due to the chitinase the midgut epithelium was not affected (Kabir et al. 2006). Fluorescence or scanning electron microscopy could be used to evaluate impact of purified chitinase activity or transgenic *Populus* on the WMTM gut.

Conclusion

We identified an AT mutant, *E8-16*, that has a negative impact on WMTM leaf consumption as well as weight gain and insect development. We have also identified the site of a T-DNA insertion and the "activated" *10s12800* gene within the mutant. We have

also begun to characterize the gene by determining where it is expressed in *Populus* tissues and identified constitutive global transcriptomic differences between *E8-16* and *Pt x Pa* wildtype tree leaf tissue. We have also conducted a forward genetics screen and identified other potential AT mutants with altered IR. Future work can still be done to identify and confirm IR mutants from the screen, characterize the *10s12800* gene and its role in IR, or explore genes identified from the microarray and how they impact insect feeding and development. These experiments may lead to new gene targets and a greater understanding of genes involved in IR in *Populus* that will lead to the development of superior trees for the forest industry.

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