Toward deregulation of genetically modified American chestnut for surface mine reclamation: Genomic mechanisms of transgene-mediated blight resistance

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Project Summary

We have been testing numerous candidate genetically modified (GM) lines of American chestnut (*Castanea dentata*) at the Powell River Project since 2014, with several showing promising levels of resistance and growth. Former surface mines represent an obvious context in which to begin restoration efforts using these GM trees, but extensive data on the environmental and ecological impacts of the transgenes is required before the US Department of Agriculture will consider their deployment outside of our current heavily monitored field trials. One of the first such pieces of data will be to show at the cellular level how a given transgene leads to resistance. As genes and their protein products function in regulatory networks that may involve thousands of other genes/proteins, assessing the risk (or lack thereof) when a transgene escapes requires an understanding of how that transgene functions in the tree, and whether it has undesirable off-target effects. One way to address this question is to leverage next generation sequencing to assess how gene regulatory networks are perturbed in the GM vs. wild-type lines. In this study, we have inoculated a number of trees in our GM field trial with *Cryphonectria parasitica* (chestnut blight) and sampled the resulting cankers for extraction of RNA. RNA-sequencing work is on-going, but we will achieve a detailed view of how the transgenes affect the rest of the gene regulatory network in these trees (that is, which genes are turned on/off and to what degree). The data arising from this project will supplement ongoing studies by collaborators toward the ultimate goal of de-regulation for one or more of these GM lines, and more extensive deployment of the trees for surface mine reclamation. Here we present some preliminary results from this study.

Introduction

The American chestnut (*Castanea dentata*) was once the dominant hardwood species in the eastern United States, representing 20-25% of forest canopy trees in the mixed mesophytic forests of the Appalachians, and serving as an important resource for people and wildlife. In the early 1900s, a fungal blight (*Cryphonectria parasitica*) was introduced from imported Japanese nursey stock that caused topkill of American chestnuts, and by the 1950s the species was functionally extirpated from its native range (Jacobs *et al.* 2013). The tree had many uses, including for sawtimber, cord wood for fuel, paper and tannin extraction, and nuts for humans, livestock and wildlife. The loss of fruiting American chestnut trees markedly reduced the carrying capacity of Southern Appalachian forests for certain wildlife species, in part because the tree was a more reliable producer of seed from year to year than other species and in part because it produced a greater quantity of seed that were larger and more nutritive than other sympatric forest tree species (Jacobs *et al.* 2013). Two parallel efforts to introduce blight resistance to American chestnut breeding populations have been undertaken. The first is a hybrid breeding strategy, in which blight resistance genes are introgressed from Chinese chestnut (*Castanea mollissima*). Chinese chestnut co-evolved with *Cryphonectria* and harbors substantial natural resistance. To achieve a population morphologically similar to native American chestnut, repeated backcrossing to American parents was undertaken following the initial hybrid cross. While this approach is promising, the current generation (B3F3 - three generations of backcrossing followed by three generations of intercrossing between the backcross families) still has substantial variation in disease resistance. The second approach to restoring this species is through genetic modification – the introduction of foreign genes (usually from Chinese chestnut (*Castanea mollissima*), a blight resistant sister-species) that have anti-fungal activity.

Over 400,000 ha have been disturbed by coal surface mining to date in Appalachia, much of which is still in need of reclamation. The extent of coal surface mining in the Appalachians, and thus land in need of reclamation, overlaps significantly with the historic range of the American chestnut. This overlap presented the opportunity to field-test GM American chestnuts for blight resistance, growth, and survival on land already in need of reforestation. Moreover, American chestnut exhibits only intermediate shade tolerance, and rapid growth when light resources are not limiting. Hence, the open sites available on many surface mines have the potential to be rapidly reclaimed with chestnut. From 2013 through 2015, we planted yearly cohorts of wild-type and GM American chestnut lines provided by our collaborator, Dr. Scott Merkle (University of Georgia) (Figure 1). As the GM trees are time consuming and expensive to propagate, we initially planted a cohort of non-GM (wild-type) trees in 2013 to assess the suitability of the site to chestnut establishment. As results from this initial test were favorable, in the subsequent years of 2014 and 2015, we planted cohorts of both wild-type and GM trees.

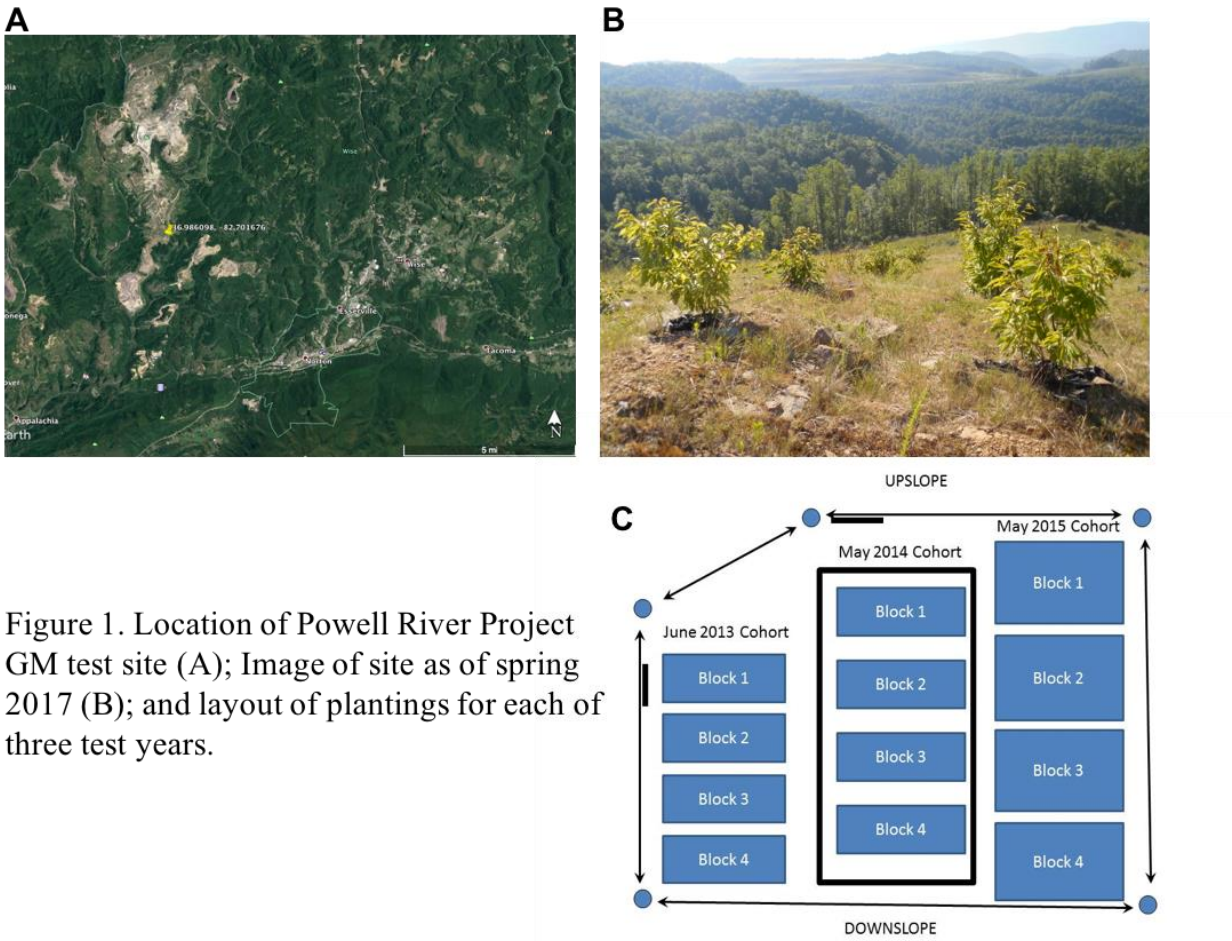

Figure 1. Location of Powell River Project GM test site (A); Image of site as of spring 2017 (B); and layout of plantings for each of three test years.

While the soils of surface mines are often difficult environments for tree establishment, preliminary data from our field trial of GM American chestnut suggest some of these lines establish and grow rapidly in this environment. Most GM lines at our PRP test site have exhibited favorable growth over the 2-3 years since planting, with height gains between 20cm and ~100cm (Figure 2). While the wild-type plants also exhibit moderate growth, they are impacted much more substantially by blight: nearly 30% of wild-type trees have already died from blight since planting began in 2013. By contrast, only 12% of GM trees from the 2014 cohort and none of the GM trees from the 2015 cohort have died from blight. These results are promising for the ultimate goal of restoring surface mines with GM chestnut.

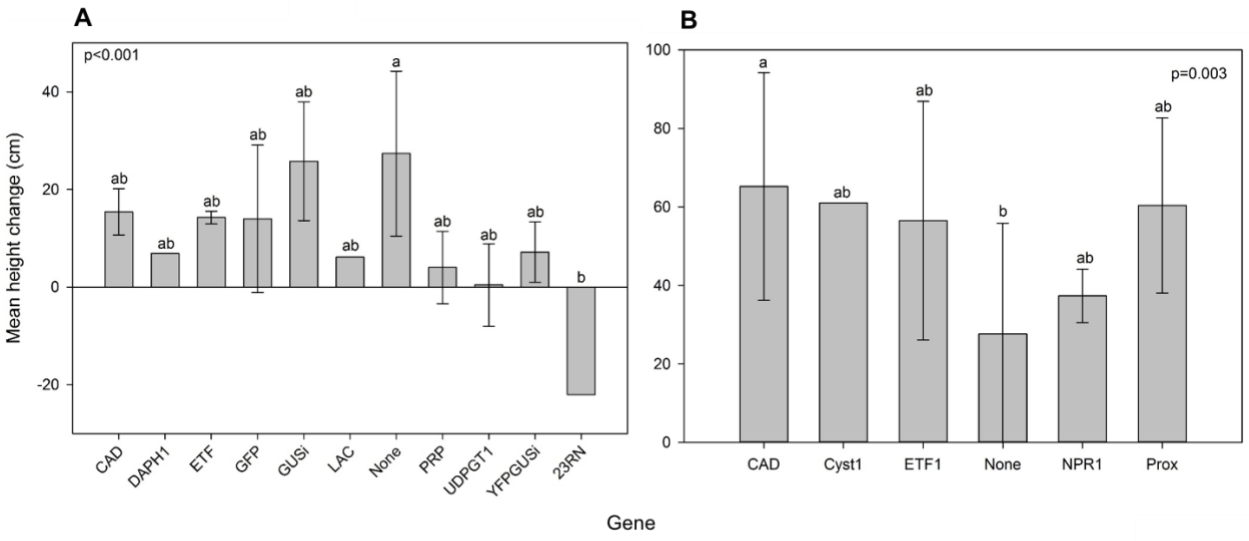

Figure 2. Mean height change for 2015 (A) and 2014 (B) cohorts as of summer 2017 for transgenic lines and wild-type plants ('none'). Note that GUSi and GFP are transgenic control (i.e., only a reporter gene with no expected effects on blight).

Project Objectives

In order to make restoration of surface mines with GM chestnut a reality, regulatory hurdles must be overcome. There are three relevant regulatory authorities for GM plants in the United States: USDA, EPA, and FDA. For our purposes, the most important is USDA, which regulates the introduction of plant pests through genetic engineering. Introduction of foreign genes into plants usually employs a method that involves also introducing DNA from *Agrobacterium tumafaciens*, which is the causative factor in crown gall disease. While the particular sequences involved in this process do not cause this disease in the resulting GM plants, they are nevertheless regulated articles. Planting such GM products without restrictions (sometimes called 'de-regulation', although they are never truly de-regulated) requires demonstrating to USDA that there is no hazard to sexually compatible species (i.e., when the transgene escapes) or the environment. While there is no 'roadmap' to follow for de-regulation, a central question for regulators will be the molecular effects of the transgenes. For example, are there pleiotropic effects beyond the focal trait (blight resistance)? What is the mode of action of the transgenes? How does the transgene interact with other genes/proteins in order to exert the favorable effects (James *et al.* 1998)? The central dogma of biology states that biological information flows from DNA (the genetic material) to protein (the molecules doing the actual 'work' in the cell) through an mRNA intermediate. By monitoring the levels of specific mRNAs in response to some stimulus, it is possible to describe the effects of a genetic modification in comparison to wild-type controls. Which genes are 'turned on'? Which genes are suppressed? What is the magnitude of these effects? We are fortunate to have at our disposal a technology that allows us to begin to address these questions, namely, RNA-Sequencing (RNA-Seq). The premise behind this technology is that by sequencing all of the mRNA molecules in a given tissue sample, we can characterize both their identity (which genes are turned on/off) and their expression levels (by how much) (Kukurba & Montgomery 2015). Given time series data, we can even model the interactions

between genes. We propose to leverage RNA-Seq technology to characterize the molecular details of transgene action in our GM American chestnut field trial, in relation to wild-type controls. These data will provide an important piece of the 'regulatory puzzle', with the goal of enabling further planting of GM chestnuts for reclamation of Appalachian surface mines, both in Virginia and neighboring states.

Methods and Procedures

The overall goal of this project was to inoculate our field trials with *Cryphonectria* and monitor global gene expression in GM and wild-type American chestnut in response to this pathogen challenge. To do this, we first secured a USDA permit to acquire and transport the parasite from The American Chestnut Foundation's Meadowview field station. On May 17, 2018, we inoculated a subset of trees at our PRP field site with the highly pathogenic isolate of *Cryphonectria parasitica*, EP155, provided by collaborators at The American Chestnut Foundation. We selected three transgenics and three non-transgenics for this study, due to availability of surviving members of certain constructs, stem size, and blight resistance data. The specific transgenics we selected were the following: CAD, Prox, and GUSi. The CAD and Prox genes reflect different hypothesized modes to pathogen resistance. For example, CAD (cinnamyl alcohol dehydrogenase) is a member of the phenylpropanoid pathway that leads to a number of defense compounds (stillbenoids, flavanoids, chalcones), but also branches to the lignin biosynthesis pathway that may be indirectly involved in defense by preventing pathogen penetration (Bhuiyan *et al.* 2009). On the other hand, Prox (peroxidase) mitigates the production of free radicals, which are known by products of *Cryphonectria* infection and can degrade biomolecules leading to cell death (Wojtaszek 1997). The GUSi construct is a transgenic control to account for any differences due to the GM process..

Inoculation followed standard procedures developed by TACF, and two replicates of each GM/wild-type were inoculated. Specifically, a fire-sterilized cork borer was used to create two circular wounds (0.4 cm diameter x 0.4 cm depth) approximately 20 cm above the root crown on the main stem (s) of each tree. Stems needed to be at least 1 cm in diameter to avoid killing the tree from the wound itself. An identical cork borer was fire-sterilized and used to cut 'plugs' of inoculum, which was plated on agar. A plug of inoculum was placed in each wound using a fire-sterilized metal spatula, with the culture facing towards the center of the stem, and masking tape was used to secure the inoculum in place. Blight symptoms/cankers were allowed to develop for one week, until 5-23-18, at which time samples were collected from treated trees by using a fire-sterilized cork-borer to collect plugs of tissue from the phloem/cambial region around both of the inoculation sites. All plugs from a single tree were composited into one sample, wrapped in foil, and flash-frozen in the field with liquid N_2. Samples were transported back to campus and stored at -80 ºC until they were processed.

RNA was extracted using Qiagen RNeasy mini kits. This step took longer than anticipated due to the degree of secondary metabolites in the sampled tissue (compared with, for example, young leaves, which are typically easier to work with), which necessitated repeating some samples with and adding additional purification and quality-control steps. Stranded mRNA libraries will be prepared at a sequencing center. mRNA samples will be barcoded and randomly assigned to pools of 12 for Illumina sequencing in a paired-end format, where each fragment is sequenced from ends. This approach improves mapping of the reads to the draft chestnut genome. One we

receive the RNA-Seq data, it will be QC-filtered and mapped to the Chinese chestnut reference genome with Tophat2 software (Kim *et al.* 2013). The alignment output will be processed by HTSeq (Anders *et al.* 2015), and differential expression among GM trees and between GM and wild-type trees will be assessed by DESeq2 (Love *et al.* 2014) using SLIM (Wang *et al.* 2011) for multiple testing corrections. The resulting data will be lists of genes that are differentially regulated both at a baseline level among GM/wild-type trees (i.e., irrespective of pathogen presence) as well as lists of genes that are differentially regulated specifically in response to the blight pathogen treatment. These data will be summarized according to functional categories of differentially regulated genes, as well as in relation to pathways specifically dysregulated in GM vs wild-type trees, which will provide insights into the mechanism by which the transgenes enable resistance to *Cryphonectria*. All data will be made publically available through deposition in a public repository (i.e., NIH GenBank), and processed differential expression data will be published along side the resulting manuscript.

Results

At this point, RNA-sequencing data has not yet been received, but we have been monitoring the inoculation sites and present some qualitative data on canker formation among the selected trees. Disease symptoms were observed on all inoculated trees, but was less severe for the transgenic plants (Figures 3, 4, and 5). The transgene that appeared to perform best was PROX, for which small cankers developed but rapidly healed. By contrast, both of the CAD trees inoculated developed more severe disease phenotypes, particularly for clone 454 (Figure 4). In addition to inoculating the trees to be included in the RNA-seq study, all other trees at both the PRP and Kentland Farm field sites were inoculated. This will allow us to better characterize the degree of resistance for the various transgenic present at these sites. We plan additional canker measurements this fall will reveal which, if any, transgenes show promise for future development as deployment lines for chestnut restoration.

Figure 3. Disease progression for clone 475 of CAD GM trees	5/23/2018	5/24/2018	7/18/2018
Tree ID# 475		No image	
Gene CAD			
Background genotype 76-5xOP			
Blight assessment notes	Cankers observed at inoculation sites, one stem dead from blight. Cankers on average, circle about 40% of stem. Overall blight symptoms on 10-25% of tree.	N.D.	Three total cankers, on average circling about 60% of the stem.
Notes	Tree leafy with no wilting/leaf browning.	Tissue sample collected.	Tree leafy with some wilting/leaf browning on ~25% of leaves.

Figure 4. Disease progression for clone 454 of CAD GM trees		5/23/2018	5/24/2018	7/18/2018
Tree ID#	454			
Gene	CAD			
Background genotype	76-5xOP			
Blight assessment notes		5 cankers observed fully encircling stem with stromata on 80%. Middle stem is dead from blight. Overall blight symptoms on ~51-80% of tree.	Some canker symptoms starting to develop around the inoculation sites.	7 cankers observed encircling on average 80% of the stem with stromata present on about 50% of cankers. New shoots formed. Overall blight symptoms on ~81+% of tree.
Notes		Tree moderately leafy with some browning/wilting on ~25% of leaves.	Tissue sample collected (photo taken before collection).	Tree moderately leafy, leaves beginning to yellow, some wilting, browning on ~40% of leaves.

PROX Hybrid Gusi

Figure 5. Blight symptoms on additional transgenic (PROX, Gusi) and non-transgenic hybrid trees. Images taken on July 18, 2018.

Benefits and Outcomes

Our data suggest that one or more of these GM lines of chestnut trees are prime candidates for restoration of surface mines with native forests, but achieving this goal requires detailed data on the mode of action of the transgenes. The proposed research will provide the first data on the molecular mechanisms governing GM chestnut resistance to the fungal blight *Cryphonectria parasitica*, and thus represent one piece of data required by regulators before these trees can be more widely planted for such restorative purposes.

Additional funding secured

In part on the basis of our work at the PRP and Kentland GM chestnut field sites, we successfully competed for a Biotechnology Risk Assessment Grant, in collaboration with

researchers at the State University of New York, Syracuse (PI: William Powell, Co-PI Jason Holliday and five additional Co-PIs at SUNY and The American Chestnut Foundation). The award amount was $500,000 (total) for a period of three years beginning September, 2018. The goal of this project is to establish long-term open-field and shelterwood trials of Oxalate Oxidase (OxO) transgenic chestnuts. These will be placed at three locations encompassing most of the native range of chestnut (Kentland Farm, VA; State College, PA; and Syracuse, NY). A variety of ecological data will be collected, including effects on belowground beneficial fungal associates, leaf litter decomposition, and effects on insect associates (especially pollinators). This work will add to the wealth of greenhouse studies that our partners at SUNY have conducted over the past decade, which show that GM chestnuts are unlikely to cause ecological harm if used for restoration. With the expectation that GM chestnuts will be 'de-regulated' in the next five years, this project will indirectly benefit surface mine reclamation efforts at the PRP and elsewhere by enabling unmonitored plantings of disease resistant chestnuts on these sites.

Publication

Once the RNA-seq data has been analyzed, we anticipate at least one peer-reviewed publication to arise from this project.

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