TEN THOUSAND CHESTNUT CHALLENGE

Progress Report

Moving Closer to 10,000 Trees

Activities undertaken between November 15, 2016 and November, 15, 2017

We have again made good progress over this year despite a few challenges. Here are some highlights – more detail on each is found below.

- Established tissue culture T1 trees from 2016 crosses, in order to propagate these trees. T1 trees are the blight resistant offspring from transgenic Darling 54 and Darling 58 American chestnut trees crossed with wild-type "mother" trees.
- Produced additional T1 trees for testing and establishing a more diverse production orchard.
- Completed several environmental impact experiments, with the final nutritional study and bumblebee feeding study to be completed by December 2017. We are currently writing the federal regulatory applications with a goal of submitting the first one for review in January 2018.
- Began upgrades of the Lafayette Road Experiment Station production greenhouse.
- Completed the first stage of a small pilot study with The American Chestnut Foundation (TACF) to cross our trees with their *Phytophthora* resistant BC3-F2 trees to see if we can stack blight and *Phytophthora* root rot resistance, prevalent in some areas of the southern range.

Established tissue culture T1 trees from 2016 crosses

Last year (2016), we produced eight T1 trees expressing the oxalate oxidase (OxO) enzyme with an equal number of full siblings without the OxO gene to be used as controls in various studies. These are uniquely valuable trees because they are produced from pollen collected from lab-produced transgenic trees, crossed with wild type American chestnuts, so they contain more genetic diversity than the original lab-produced trees. This year (2017), buds from the blight resistant T1 trees were grafted and the shoots that grew were harvested and used to establish them in tissue culture. The purpose of tissue culture was to produce large numbers of trees from the eight T1 offspring.

Seven cultures were established representing trees with Darling 54 or Darling 58 as the pollinator (father) and three wild-type mother trees. In addition to the increased genetic diversity, tissue culture lines from these T1 trees are fresher cultures so they grow and multiply better than older cultures. We plan to establish production orchards with these trees as well as use them to produce pollen for further outcrossing to wild-type American chestnut trees.

Produced T1 trees for testing and an even more diverse orchard establishment

Throughout 2017, more transgenic pollen was produced from the blight resistant Darling 54 and Darling 58 trees in our high-light growth chamber and greenhouse, and stored frozen. In late June, it was thawed and used to pollinate wild-type "mother" trees that had their female flowers (burs) previously bagged to prevent unwanted pollinations. Slides, brushes, and dipping pollination methods were tested to see which was most efficient. Using slides appeared to be the most efficient pollination method when the amount of pollen is the limiting factor, as is the case with transgenic pollen. The pollinated burs were bagged with wire mesh screens to protect them from squirrels and blue jays.

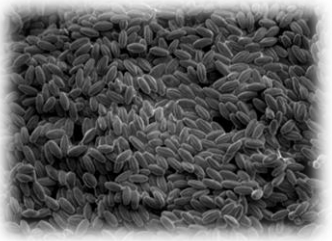
The nuts were harvested in the fall and sorted. A total of 181 viable nuts were collected. Forty were cored and tested for OxO expression and then ground up for nutritional testing. Approximately 60% tested positive for OxO, which is close to the 50% expected to be positive. Core samples were also tested to determine the concentration of OxO enzyme for the regulatory review.

We faced another challenge this year when our nearly 50 year-old refrigerated cold room malfunctioned one weekend and froze the stored nuts. We expect some nuts will survive, but we won't know how many until after they are cold stratified for three or more months. Instead of producing approximately 90 T1 trees this year as hoped, production will likely be much lower. To prevent a malfunction in the future, we are installing sensors that will send text message alerts. As funding allows, we would like to purchase new equipment to replace the old. Compared to 2016, we expect to produce more T1 trees in 2017 despite the malfunction.

Completing experiments for the regulatory review process

We have completed many experiments needed for the regulatory review. These include genomic location of the oxalate oxidase (OxO) transgenes, level of OxO expression, analysis for allergens, nutrient content of nuts (mentioned above), feeding studies with terrestrial and aquatic insects, wood frog tadpole feeding on chestnut leaves, leaf decomposition, seed bank germination in leaf litter, mycorrhizal (beneficial fungi) colonization of the roots, and a compilation of related literature to answer the regulators' questions. Results so far overwhelmingly indicate the transgenic American chestnut trees are not significantly different, except for blight resistance, than the wild-type trees or other chestnut trees produced by breeding.

Our final experiment this year involves feeding bumblebees chestnut pollen with different concentrations of OxO. To do this, we recruited a honeybee hive equipped with a pollen basket collector to collect chestnut pollen. The hive was set up near our flowering non-transgenic mother trees and the bees did the work of collecting chestnut pollen. Our graduate student Dakota Matthews examined the resulting pollen with a scanning electron microscope to identify which pollen was primarily from



chestnuts. This pollen was then mixed with known quantities of OxO, to simulate what we would see on larger flowering transgenic chestnut trees, and this treated pollen is currently being fed to bees. We are looking for any differences in pollen usage or survival between the bumblebees eating the OxO. We don't expect to see any differences between bees that eat the OxO and those that do not.

If all goes as expected, we intend to incorporate data from these experiments, finish writing the large regulatory applications, and submit them for review in January 2018.

Upgrading of the Lafayette Road Experiment Station production greenhouse

As we add tree production to our research, we will need additional facilities and equipment to reach our goal of 10,000 chestnut trees. ESF has given us additional greenhouse space for production at the Lafayette Road Experiment Station. This old greenhouse needs several repairs and upgrades. ESF is providing new heaters for this space and helping with the installation of new vents. Donations from the 10,000 Chestnut Challenge and other sources have allowed us to purchase and install state-of-the-art lighting and thermostat-controlled vents. The goal is to use these greenhouses for overwintering dormant trees and for growing our plantlets and seedlings larger before taking them to the field.

Pilot study with The American Chestnut Foundation (TACF) to cross our trees with their Phytophthora resistant BC3-F2 trees

In some, but not all, areas of the southern American chestnut range there is a second pathogen that can kill American chestnut trees. *Phytophthora cinnamomi* causes root rot, which can be even more damaging than blight, as it kills roots and doesn't allow trees to re-sprout. Therefore, to effectively restore the American chestnut tree to its full natural range, we need to combine blight resistance with *Phytophthora* resistance.

We are working toward this stacked resistance in two ways. First, we are testing genes using genetic engineering, as we did with blight resistance. One promising gene we are working with comes from grapes. It will take a year or two to see if this gene will enhance resistance to *Phytophthora*. A second way is to breed in *Phytophthora* resistance using some of the TACF BC3-F2 trees that inherited this resistance from a Chinese chestnut parent tree. The advantage of breeding is that this method of gene transfer is exempt from regulatory review, saving time and money. Therefore we started a pilot project where we sent pollen from our transgenic blight resistant trees to the TACF Meadowview farm, where they performed some crosses. This year they harvested 106 nuts, of which we expect about half should have the OxO gene. After cold stratification, these will be grown and tested for OxO expression, blight resistance, *Phytophthora* resistance, and used for further crosses. The breeding will take several years to complete, but it will be accelerated using our early pollen production methods.