Co-Directors, Drs Bill Powell and Chuck Maynard have set up a project to rescue the American chestnut tree from extinction and restore it to its former glory as a keystone species of forests in the Eastern US.

Avoiding extinction

From what context has the American Chestnut Research and Restoration Project evolved? How do you aim to redefine America’s forests?

CM: The American chestnut (Castanea dentata) was valued for its nuts and wood and considered an American heritage tree species. However, this was all lost due to the introduction of an exotic pathogen, Cryphonectria parasitica over a century ago, which causes chestnut blight. Presently, we don’t intend to redefine any forests, but to take the first steps to restore the forest to what it was before the blight.

To what extent is blight still a problem in the US?

BP: Today a remnant population of American chestnut trees is surviving mainly as stump sprouts that rarely reach an age to reproduce. Blight is here to stay because it can survive on the bark of many oak species, causing little or no damage to these trees. Therefore, to restore the American chestnut, resistance to blight is key.

Additionally, in the southern part of its range, the American chestnut is also being killed by Phytophthora root rot. Thus, our colleagues and we are also looking for resistance-enhancing genes to this important pathogen.

Could you both introduce your roles within the Project and explain how your experiences led to an interest in forestry? In what ways do your skills complement each other?

BP: I started working with chestnut blight in 1983 as a graduate student with Dr Neal Van Alfen. My project investigated a double-stranded RNA virus in the fungus that causes it to become hypovirulent (reduced virulence). When I came to the State University of New York, College of Environmental Science and Forestry (SUNY-ESF), I met Chuck and we quickly formed a collaboration to develop blight-resistant trees. I use my skills in molecular biology and plant pathology to complement Chuck’s skills in plant tissue culture and forestry.

CM: We also complement each other by providing better access to other scientists in our respective departments than we would have if the project was based in just one department. Bill has recruited a plant physiologist with expertise in pollen viability, an expert mycologist to study mycorrhizal root development, a forest entomologist to study the insects that feed on chestnut and an entire team of restoration ecologists. I have brought a silviculturist onto the team who helped us set up a realistic test of how well our trees would perform if planted into openings in existing forests, a statistician to help with field plot design and a soil scientist to study how well the foliage from our blight-resistant trees decomposes.

What challenges have you been faced with, and how have you overcome them?

BP: Early on, the biggest challenge was that many people didn’t think blight resistance could be achieved through genetic engineering; it was a fool’s errand and too risky to support. This made it difficult to obtain traditional grant funding. Fortunately, we did receive funding through non-traditional places such as the New York chapter of The American Chestnut Foundation, New York State legislature and a company called ArborGen. Later, as we started showing success, more traditional funding sources started supporting the chestnut project and we hope that will continue because there is still much work to be done.

CM: The next biggest challenge has been to find and train a highly skilled and dedicated team of people capable of doing the research to develop the protocols. We have been incredibly fortunate to work with some amazingly talented people.

Looking back at your results so far, which have been most promising or even surprising?

BP: The most satisfying was the small stem assays where we demonstrate that our newest trees are as (if not more) resistant than the Chinese chestnut controls. One surprising result was from our recent metabolomics studies, showing that we are making much smaller changes to the metabolites using genetic engineering than is being obtained through traditional hybrid breeding.

CM: I agree with Bill. His two-minute video of four little chestnut trees encapsulated a 25-year effort. Another highlight was planting 10 chestnut trees at the New York Botanical Garden. This site was a stone’s throw away from where the chestnut blight was first identified in 1904 in the Bronx Zoo.
By improving on current plant transformation techniques and testing disease resistance-enhancing genes, researchers at the State University of New York’s College of Environmental Science and Forestry have successfully created a blight-resistant American chestnut.

**THE AMERICAN CHESTNUT** was an iconic species of eastern US forests before the 20th Century. These trees feature strongly in American heritage, immortalised in national songs and poems. Chestnuts were also an important food source, as well as a fast-growing source of naturally rot-resistant lumber – about half to three-quarters as strong as oak, but only about 60 per cent of the weight. However, as Asian chestnuts were introduced into North America commercially as an orchard tree so was chestnut blight, caused by the fungus *Cryphonectria parasitica*. As a result, the American chestnut population was reduced from an important part of the forest ecosystem to a small, functionally extinct shrub. Within 50 years, the blight fungus had spread through the chestnut range from the coast of Maine in the north to that of Georgia in the south, and had killed 3-5 billion American chestnuts, destroying the food source for animals such as the now extinct Carolina parakeet. It has been described as one of the greatest ecological disasters of the 20th Century.

Currently, two main programmes aim to develop blight-resistant trees. The first is breeding the American chestnut with the blight-resistant Chinese chestnut and then breeding the resulting trees (backcross) with the American chestnut in order to produce trees that are mostly American chestnut, but also have the favourable blight-resistant characteristics of the Chinese chestnut. However, it is a lengthy process to breed out all unwanted Chinese chestnut traits. Drs Bill Powell and Chuck Maynard, co-Directors of the American Chestnut Research and Restoration Project and their team decided to follow the second method and introduce blight-resistance genes into American chestnut trees. 110 years after the first discovery of blight, the team has developed resistant transgenic chestnut trees.

**AGROBACTERIUM: THE NATURAL GENETIC ENGINEER**

*Agrobacterium tumefaciens* is the most commonly used vector for introducing new genes into plant cells. In nature, it can attach to healthy cells near the edges of small wounds on the root collar of many plants. It then causes pores to form in the plant’s cell wall. Through these pores, it can insert genes into the cell, which are then integrated into the plant genome allowing the genes to be expressed as proteins and inherited by daughter cells. These gene products allow the bacterium to ‘control’ the plant cell and cause it to divide rapidly to produce a gall and plenty of nutrient-producing cells for the bacteria to thrive. This natural process has been harnessed for research purposes by replacing the disease-causing genes in ‘wild’ *A. tumefaciens* with the genes that scientists want to insert into the chromosomes of the plant, thereby transforming the cell. Through this method, genes can be introduced into a plant from virtually any species.

Powell’s team used a strain of *Agrobacterium* with blight-resistance genes encoded, as well as a marker to identify which cells were expressing the inserted DNA. This strain was grown overnight in a liquid medium, and then mixed with chestnut embryos for approximately one hour. The thoroughly coated embryos were transferred to desiccation plates and cultured for several days, allowing sufficient time for the bacteria to insert genes into the embryos. Desiccation plates are similar to Petri plates, but the watery gel-like medium used in the standard protocol is replaced with a slightly moistened absorbent paper disc. These discs gradually dry, causing the *Agrobacterium* to adhere more tightly to the embryos, thereby increasing transformation frequency, an improvement on the standard transformation protocol.

The embryos were subsequently mixed with a cocktail of antibiotics to kill any remaining *Agrobacterium*, as well as any plant cells that have not taken up the new DNA and grown in a periodic immersion bioreactor – another deviation from the *Agrobacterium* transformation protocols that many other labs use. The bioreactor immersed the growing embryos in the liquid medium at intervals. Remaining embryos were then grown into transgenic, blight-resistant trees. Maynard outlines the importance of their protocol development: “It has taken us 17 years to develop a reliable system for transforming American chestnut. Now that we have it, we can go from a tiny piece of DNA and a few Petri plates of somatic embryos to a transgenic plant, ready in to go to the field in approximately one year.”

**Biotechnology, such as the project’s improved Agrobacterium-mediated transformation, has the potential to play a bigger role in restoration projects**

The team successfully inserted a gene from a species of wheat into the American chestnut trees. This wheat gene was selected as it is already a food source. A gene construct with the wheat oxalate oxidase gene was created. The enzyme produced will detoxify the damaging oxalic acid produced by the blight fungus, protecting the tree. This does not kill the fungus, but disarms it, changing its lifestyle from a pathogen to a saprophyte. The gene was inserted using the Agrobacterium-mediated transformation and successfully produced the American chestnut ‘Darling 4’, which had intermediate levels of blight resistance between that of the American and Chinese chestnut. The researchers then demonstrated a correlation between the level of blight resistance and the expression of the oxalate oxidase gene, with newer ‘Darling’ events showing levels of blight resistance equal to and maybe even higher than Chinese chestnut.

The team has also been studying many putative resistance-enhancing genes from Chinese chestnut and have identified a laccase-like gene and a proline-rich protein gene that could enhance blight resistance to intermediate levels if used to transform American chestnut embryos. Creating the transgenic trees makes it possible to specifically introduce blight resistance without affecting the desirable timber-type characteristics of the American chestnut.

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Chinese chestnut induced to produce catkins & burs in as little as 6 months under high-light treatments. American chestnut (not shown) only produced catkins and took a minimum of 11 months.
Aseptic removal of immature embryos, the first step in establishing somatic embryo cultures for use in transformations experiments.

REBUILDING THE CHESTNUT FOREST

The next step in the realisation of Powell and Maynard’s vision of a restored chestnut population is to provide data to three federal regulatory agencies, the United States Department of Agriculture (USDA), Environmental Protection Agency (EPA) and Food and Drug Administration (FDA), showing that their transgenic chestnuts are safe for introduction into American forests. Progressing through the regulatory evaluation could take several years but, once complete, they can begin their restoration programme.

After regulatory approval, the first step of the restoration programme will be to increase the diversity of the transgenic chestnuts, rather than simply introducing the transgenic plants straight into the population as they are. To do this, the group plans to ‘introgress’ the genes into the population. First by pollinating ‘mother’ trees maintained by The American Chestnut Foundation and later pollinating isolated flowering chestnuts from the wild, blight-susceptible population with pollen from the transgenic chestnuts. The seedlings produced will be screened for resistance and sent out to willing members of the American Chestnut Foundation in New York. Maynard explains how these benefactors enable the beginning of the restoration: “These members have already planted individual non-transgenic trees in their yards. By the time we have seedlings ready to ship, the members’ trees will be flowering, so these will be used as mother trees”.

The second step will be to encourage another outcross to wild type chestnut trees by asking that the cooperators plant the seeds they collect from these mother trees far enough apart so they cannot cross-pollinate, but near one or two native trees that they can cross with. These trees and their offspring will be monitored for several years. “From that point on, we plan to let the multitude of natural seed dispersers (squirrels, jays, chipmunks, etc.) do the distribution work for us,” Maynard elaborates.

BIOTECHNOLOGY AS A TOOL FOR ENVIRONMENTAL RESTORATION

The expected success of the Project links biotechnological approaches more closely with environmental restoration projects. This is a new paradigm that may provide an additional tool to saving threatened plant species. Biotechnology, such as the project’s improved Agrobacterium-mediated transformation and gene selection, has the potential to play a bigger role in restoration projects, augmenting more traditional methods such as breeding programmes. “We are demonstrating how an important tree species can be saved through genetic engineering,” Powell explains. “These same techniques can be used to help other trees, such as the ash and hemlock, which are being devastated by pests and pathogens.” Therefore, The American Chestnut Restoration Project, could potentially not only save one species from extinction, but also set a new standard for environmental restoration.

First transgenic American chestnut seeds produced by crossing pollen produced under high-light treatments from a transgenic American chestnut and crossed to a wild type American chestnut in the field under controlled conditions.

AMERICAN CHESTNUT RESEARCH AND RESTORATION PROJECT

OBJECTIVES

The mission of the American Chestnut Research and Restoration Center is to conduct basic and applied research that will lead to the development of blight-resistant American chestnut trees (Castanea dentata). The goal is to eventually reintroduce a population of these trees back into forest ecosystems and use the knowledge gained with other threatened tree species.

KEY COLLABORATORS/PARTNERS

Dr Scott Merkle; Dr Joe Nairn, University of Georgia; Dr Jason Holliday, Virginia Tech • Dr Tim Tschaplinski, Oak Ridge National Labs • Dr Bern Sweeney, Stroud Water Research Center • Dr Tom Horton; Dr Dylan Parry; Dr Chris Nowak; Dr Russ Briggs; Dr Don Leopold, State University of New York, USA

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DR CHARLES MAYNARD is Professor of Forest Genetics at the State University of New York, College of Environmental Science and Forestry. Along with Powell, he has been a co-Director of the American Chestnut Research and Restoration Project since 1990. He received his BS, MS and PhD from Iowa State University.