

Diversifying our investment portfolio

Bonds - low risk, steady, but slow return - best x best crosses among our most blight resistant backcross trees- Use controlled pollinations to intercross a subset of the most blight resistant backcross trees. Plant the progeny in at least one regionalized seed orchard per region (i.e. intercross southern trees, mid-atlantic, north central, and new england trees). Inoculate and select the most resistant trees and continue to perform recurrent selection to enhance blight resistance while also selecting trees that maximize American chestnut genome.

Why continue traditional breeding at reduced scale? Continuing the breeding program provides us with a non-GMO approach towards blight resistance. We do not have permission yet to plant Darling 58 offspring on Forest Service land and lands certified by the Forest Stewardship Council and the Sustain Forestry Initiative. Given the uncertain social and legal status of Darling 58 at the moment, it is prudent focus a small amount of our organizational effort continuing to advance our most blight resistant material in the traditional breeding program

Index fund - Combine the best of all existing approaches - Breed Darling 58 and other OxO transformed trees with backcross trees and wild type Americans conserved in GCOs. Using backcross trees and existing wild trees in GCOs parents shaves decades off the multigenerational diversification process as compared to starting from scratch now on conserving wild trees for outcrossing to transgenic trees. Breeding OxO trees with PRR resistant backcross trees enables us to generate trees with resistance to both major diseases that wiped out American chestnut. There is still some uncertainty about the durability of OxO resistance when it is bred into diverse genetic backgrounds and planted in contrasting environments. Breeding OxO trees with our most blight resistant backcross trees enables us to stack resistance from Chinese chestnut along with OxO potentially providing more robust resistance than with either OxO bred with wild type or backcross trees bred with themselves.

Venture capital - high risk, high cost, potentially high reward - pursue gene editing on 500 wild type American chestnuts to simultaneously enhance resistance to both chestnut blight and *Phytophthora* root rot in a genetically diverse population. Performing the same edits in multiple genetically diverse American chestnut trees potentially circumvents the need for multiple generations of outcrossing to wild type to enhance diversity of the populations. However, the gene discovery and validation steps are potentially time and resource intensive.

There are a number of steps required to open up this pathway. 1. Complete the reference genome for Chinese chestnut (American chestnut is done). 2. Perform correlative approaches to discover candidate genes for resistance. Current approaches include A. RNA seq time courses to compare gene expression in American and Chinese chestnut in blight infected stems and root rot infected roots. B. QTL mapping for resistance in hybrid populations. C. Compare genomes of resistant and susceptible species, look for signatures of purifying or balancing selection that are present in resistant but not susceptible *Castanea* species. Combining A. B. and C. approaches is what we are hiring a post doc to do. We likely will need to perform additional research to better understand the components of blight resistance and the underlying genes associated with these components. For example, can we identify metabolites that inhibit the growth of the fungus in Chinese chestnut that are absent in American chestnut? Is Chinese chestnut less sensitive and therefore has less cell death in the presence of oxalic acid than American chestnut?

Once we have candidate genes discovered, there are a number of options to confirm their function in resistance

1. Knock them out in Chinese chestnut with CRISPR and determine if Chinese chestnut becomes more susceptible
2. Insert them into American chestnut and see if resistance is enhanced.
3. Edit American chestnut alleles to be like Chinese chestnut alleles and determine if resistance is enhanced.
4. Insert large QTL regions from Chinese chestnut into American chestnut and determine if resistance is enhanced (circumvents need to know the exact genes and allows for structural variants to be incorporated in American chestnut genome).

Questions for discussion of gene editing:

1. Do we knock out/in single genes and test for their resistance effects? Seeing a phenotypic effect may be challenging given that blight resistance is multi-genic and each change would likely only have subtle effects. Is there an intelligent way to multiplex multiple edits and test for the effects in combination with other edits?
2. If we make multiple edits at different locations in the genome are we tied to a. to making the same edits homozygous in multiple trees (transforming 500 trees is an enormous task)? Or do we breed these edits into multiple wild type American chestnut breeding lines? If we use breeding, we would need screen large progeny sets to select a small subset of offspring that inherited all genes.
3. Are we better off taking guesses about which genes confer resistance based on what has been discovered in model systems? And/or should we be pursuing foundational research to understand genes/mechanisms of blight and PRR resistance

Steps to prepare us for all three paths outlined above:

Establish common garden experiments to evaluate long-term blight resistance of Darling 58 progeny. Compare resistance and growth characteristics of progeny of Darling 58 x wild type and Darling 58 x blight resistant backcross trees. Make crosses between Darling 58 x PRR resistant backcross trees. Select for PRR resistance with genetic markers and plant heterozygous progeny in *P. cinnamomi* free sites in hopes that the trees will survive long enough to flower so we can intercross and make PRR resistance homozygous. Plant another subset of Darling 58 x PRR resistant backcross progeny at sites with *P. cinnamomi* to evaluate blight and PRR resistance of these trees in the presence of both diseases.

Increase throughput of OxO testing - Develop a high throughput method to test seed for the inheritance of OxO. Todd from Corteva recommended we work with a company to develop test strips that change color indicating OxO inheritance via antibodies for OxO)

Account for genetic diversity and disease resistance captured in the traditional breeding program

Figure out the geographic origins of wild trees with progeny that are alive now in our backcross and germplasm conservation orchards. Use phenotype and genotype data for the existing trees to make selections for blight and PRR resistance for the index fund approach. Generate whole genome sequence data on 384 backcross

trees, compare adaptive allelic diversity in BC populations to wild type populations to figure out adaptive alleles and geographic regions are underrepresented in our current program. Target future germplasm conservation to these areas.

Finish and publish genomes and correlative resistance gene discovery steps

Improve tissue culture and gene editing capability

- Develop a protocol to clone Chinese chestnut via somatic embryogenesis
- Develop CRISPR protocols to knock out genes in Chinese chestnut
- Optimize current SE techniques for American chestnut to increase efficiency of cloning diverse American chestnuts
- Develop for gain of function mutations in American chestnut, develop CRISPR knock-in, gene expression enhancement, base editing capacity for American chestnut.

Ramp up our efforts in American chestnut propagation for germplasm conservation

- Compare adaptive diversity in American chestnut BC populations and compare adaptive diversity in wild type populations. Figure out what subpopulations are under represented to target for future American chestnut propagation and germplasm conservation
- Develop a streamlined protocol to conserve and propagate wild type American chestnuts to move them to the forest into orchards. We need immature embryos as our starting material for genetic transformation
- Find funding to dedicate staff time for American propagation
- Get permit to use super donor strains to keep Americans alive in GCOS.
- Take inventory of wild type American trees we have in GCOs and that have been cloned by Scott.