Applying American chestnut biotechnology approaches for the conservation of Ozark chinquapin

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Introduction

The necrotrophic pathogen *Cryphonectria parasitica* (Murr.) Barr. (causal agent of chestnut blight) left the American chestnut (*Castanea dentata* (Marshall) Borkh.) functionally extinct in the 20th century. This host-pathogen interaction has been studied for decades, and as a result, several laboratory and field techniques are available to help bring back this tree [1]. A blight-tolerant American chestnut (Darling 58) was developed by adding a gene from wheat encoding for a detoxifying enzyme, oxalate oxidase (*OxO*), to counter the main virulence factor of the pathogen [2]. With the close deregulation of Darling 58 (D58) by the U.S. regulatory entities [3], the next logical step is to apply the knowledge from the American chestnut to other important forestry species severely impacted by the chestnut blight, such as the Ozark chinquapin (*Castanea ozarkensis* Ashe) [4].

Breeding

Controlled pollinations with transgenic chestnut's pollen

→ Transgenic American chestnuts were placed in high-light growth chambers to induce early flowering[5]. Pollen was collected in microscope slides and used fresh or stored at -80˚C until needed.

→ Ozark chinquapin female flowers were pollinated (A,B) & bagged (C).

→ Nuts from 2021 crosses were harvested (D) and stored at 4˚C before planting.

→ Leaf discs were collected from 2 months-old seedlings (E) to test for OxO presence - histochemical assay[6] (F).

Table 1. Brief summary of the number of pollen types and Ozark chinquapin mother trees used during pollinations.

Pollination year	Number of pollen types	Number of mother trees
2021	2 T_2 D58 American 1 T_1 D58 American x Allegheny Chinquapin	10 (4 years old)
2022	5 T_2 D58 American 1 T_1 DarWin American	19 (5 and 6 years old)

T_1, T_2: First and second transgenic generation progeny, respectively.
D58 American: *OxO* driven by 35s constitutive promotor; DarWin American: *OxO* driven by Win wound inducible promotor.

First results and perspectives

Table 2. Brief summary of the results from the 2021 crosses.

Flowers	Burs	Nuts	Planted	Germinated	**OxO +**	**OxO -**	Untested
977	248	209	205	121	**55**	**58**	8

~21% of pollinated flowers produced nuts

~50% gene inheritance as previously reported[7]

→ 26 OxO^+ and 26 OxO^- hybrids were planted in a regulated field for morphological comparison (G).

→ **5325 flowers** were pollinated in 2022.

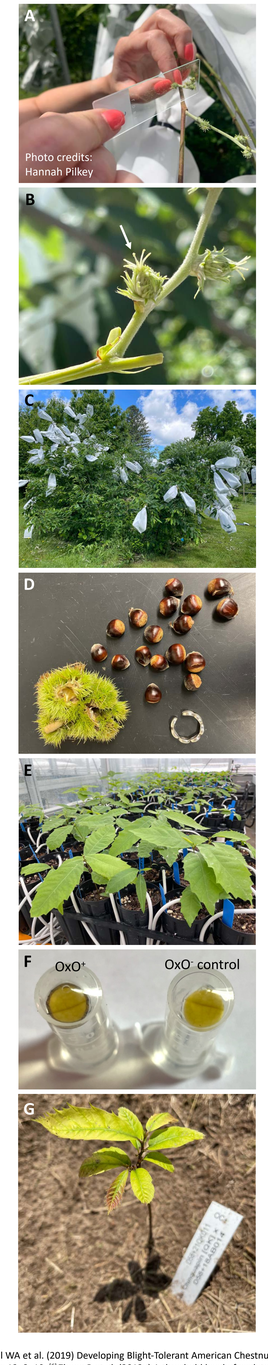
→ Approximately **1100 nuts are expected** if we consider the previous year's flower/nut rate (21%).

Future goals

→ Harvest 2022 offspring and test for OxO presence.

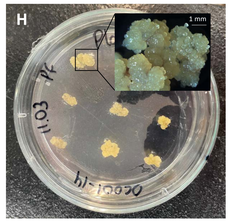
→ Phenotyping for blight tolerance and analysis of OxO expression levels in 2021 and 2022 OxO^+ offspring.

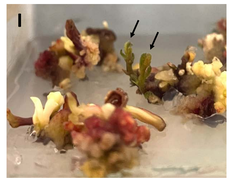
→ Induce rapid pollen production in OxO^+ D58 x Ozark chinquapin F_1T_3 hybrids: backcrosses to restore the Ozark chinquapin phenotype.

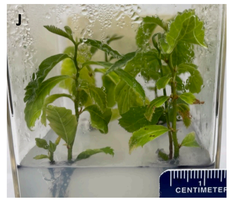


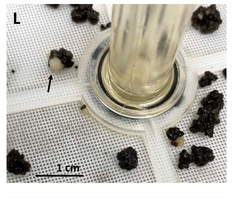
Breeding and **genetic transformation** methods developed for the American chestnut could be used to develop a **blight-tolerant Ozark chinquapin**.

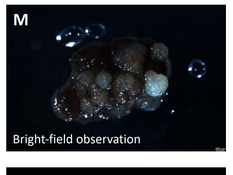
Genetic transformation











In vitro production pipeline

→ Ozark chinquapin embryo lines: OC001-14, OC005-13 (kindly provided by Dr. Scott Merkle, University of Georgia).

→ American chestnut *in vitro* culture protocols were tested (qualitative analysis): embryo multiplication and regeneration into shoots[8], shoot multiplication, elongation, and rooting[9].

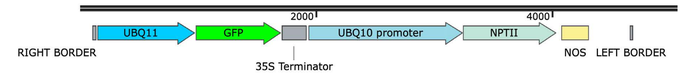
Agrobacterium-mediated transformation

→ Vector: **pFHI-GFP** (map below); green fluorescent protein (GFP) reporter gene.

→ Two weeks old embryo cultures: 266 mg OC001-14; 293 mg OC005-13.

→ Transformation: embryo co-culture with AGL-1 strain of *Agrobacterium* for 1h; place embryos in desiccation plates for 48h[10].

→ Selection: periodic flooding with selective medium in RITA® bioreactors for 8 weeks[10] .



Results

→ Embryo multiplication (H) and regeneration into shoots (I; arrows), shoot multiplication, elongation (J), and rooting (K) were **achieved** with OC001-14 genotype. OC005-13 embryos were multiplied but did not regenerate.

→ Seventeen OC001-14 events survived selection (L; arrow) and expressed the GFP reporter gene (M,N). No transformants were obtained with 0C005-13.

→ Transformants lost fluorescence over time: putative transient expression.

Future goals

→ Optimize transformation protocol: stable gene expression.

→ Obtain OxO transformants.

→ Increase background genetic diversity by transforming several somatic embryo genotypes.

References: [1] Fernandes P et al. (2022) European and American chestnuts: An overview of the main threats and control efforts. *Front. Plant Sci.* 13:951844; [2] Powell WA et al. (2019) Developing Blight-Tolerant American Chestnut Trees. Cold Spring Harbor Perspectives in Biology. 11:a034587; [3] Newhouse A et al. (2020) Petition for Determination of Nonregulated Status for Blight-Tolerant Darling 58 American Chestnut (*Castanea dentata*). https: //www.regulations.gov/document/APHIS-2020-0030-0002; [4] Anagnostakis SL (1987) Chestnut Blight: The Classical Problem of an Introduced Pathogen. Mycologia. 79:23; [5] Baier K et al. (2012) Early flowering in chestnut species induced under high dose light in growth chambers. *J. Am. Chestnut. Foundation* 12, 8–10. [6] Zhang B et al. (2013a) A threshold level of oxalate oxidase transgene expression reduces *Cryphonectria parasitica*-induced necrosis in a transgenic American chestnut (*Castanea dentata*) leaf bioassay. *Transgenic. Res.* 22, 973–982; [7] Westbrook JW et al. (2020a) A plan to diversify a transgenic blight-tolerant American chestnut population using citizen science. *Plants People Planet* 2, 84–95; [8] Maynard CA et al. (2015) Chestnut, American (*Castanea dentata* (Marsh.) Borkh.). In *Agrobacterium Protocols*; Methods in Mol Biol; Springer: NY, USA; pp. 143–161; [9] Oakes AD et al. (2020) Improving Ex Vitro Rooting and Acclimatization Techniques for Micropropagated American Chestnut. Journal of Environmental Horticulture. 38:149–157; [10] McGuigan L et al. (2020) Transformation of American chestnut (*Castanea dentata* (marsh.) borkh) using RITA® temporary immersion bioreactors and we vitro containers. Forests. 11:1–15.