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 (DS8) 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

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

<table>
<thead>
<tr>
<th>Pollination year</th>
<th>Number of pollen types</th>
<th>Number of mother trees</th>
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</thead>
<tbody>
<tr>
<td>2021</td>
<td>2 T1 OxO American</td>
<td>10 (4 years old)</td>
</tr>
<tr>
<td></td>
<td>1 T1 D58 American</td>
<td>19 (s and 6 years old)</td>
</tr>
<tr>
<td>2022</td>
<td>5 T1 D58 American</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 T1 DarWin American</td>
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</tbody>
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~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 F1 hybrids: backcrosses to restore the Ozark chinquapin phenotype.

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 [4], shoot multiplication, elongation, and rooting [9].

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Agrobacterium-mediated transformation

→ Vector: pHII-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 OC005-13.

→ 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.