# Speed breeding transgenic American chestnut trees toward restoration

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## Abstract

The American chestnut (*Castanea dentata*) was a dominant, foundational forest canopy tree in eastern North America until an imported chestnut blight (caused by *Cryphonectria parasitica*) rendered it functionally extinct across its native range. Biotechnological approaches have the potential to help restore the species, but field-based breeding advances are hampered by long generation times, ≤50% transgene inheritance, and regulatory restrictions on outdoor breeding of transgenic trees. Self-incompatibility and flowering phenology further limit generational advances and field testing of chestnuts. Our work here demonstrates that long generational times and field constraints can be circumvented by producing both male and receptive female flowers in controlled indoor environments. Additionally, we developed an embryo rescue protocol for both indoor and field conditions, in which developing embryos can be extracted and micropropagated from immature seeds between 6- and 8-weeks post pollination. These advances have enabled production of the first homozygous transgenic American chestnuts, which have produced pollen that was used for outdoor controlled pollinations and yielded nearly 100% transgene inheritance by offspring. This work also provides event-specific DNA markers to differentiate transgenic chestnut lines and identify homozygous individuals. We demonstrate that an obligate outcrossing forest tree can reach sexual maturity rapidly in controlled, indoor environments. When coupled with genomic analyses and other biotechnological advances, this procedure could facilitate the reintroduction of this iconic species.

# Introduction

The American chestnut (*Castanea dentata* [Marsh.] Borkh.) was once an ecologically, economically, and culturally significant species of eastern North American forests (United States Forest Service 1971). In the late 1800s, the invasive chestnut blight fungus (*Cryphonectria parasitica* [Murr.] Barr.) was introduced in New York and within a few decades, billions of chestnut trees succumbed to pathogen infection (Hepting 1974). This has been described as the “greatest ecological disaster since the ice age” (Freinkel 2004). Although American chestnuts are considered functionally extinct, a portion of the chestnut population survives due to the tree’s ability to asexually resprout from the root collar after blight kills it above ground (Stillwell et al. 2003). Numerous programs have been working to conserve and restore the American chestnut for over a century. One method of enhancing blight tolerance in chestnuts has been achieved through genetically engineering American chestnut via *Agrobacterium*-mediated transformation (Maynard et al. 2015). The most promising transgenic chestnut trees thus far express an oxalate oxidase (*OxO*) gene derived from wheat, which neutralizes the toxic oxalic acid delivered by the fungus (Liang et al. 2001; Zhang et al. 2013). Trees with the *OxO* transgene are collectively referred to as ‘Darling’ (Powell et al. 2019). Previously described Darling chestnuts include Darling 4, a two-copy transgenic line expressing *OxO* under the *VspB* vascular promoter (Newhouse et al. 2014), and Darling 54 and Darling 58, which are single-copy transgenic lines (Powell et al. 2020, Steiner et al. 2017) with OxO expressed under the CaMV35S constitutive promoter (Guilley et al. 1982).

While transgenic American chestnuts can be produced through micropropagation and acclimatization (Oakes et al. 2020), clonal propagation is not ideal for restoration goals: breeding blight-tolerant Darling trees with surviving wild chestnut trees is necessary to conserve wild-type germplasm, introgress the *OxO* gene, and increase genetic diversity (Westbrook et al. 2020). Transgenic American chestnut pollen was first used for pollinations in 2011, and Darling 54 pollen has been in use since 2016. However, progress has been hindered by the reproductive traits of the chestnut. Like many long-lived tree species, chestnuts are slow to reproduce. American chestnuts typically produce staminate (male) flowers bearing pollen in three to five years (Andrade et al. 2009), and it can take seven to ten years for trees to produce pistillate (female) flowers (Cook and Forest 1979). This can stretch to twenty or more years for trees growing in understory conditions (Paillet and Rutter 1989). Chestnuts are also largely self-incompatible, requiring outcrossing with other individual trees for successful fertilization (McKay 1942; Burnham 1988). Thus, when starting with two similar-aged field-grown seedlings, it can take upwards of seven years to intercross and produce viable offspring (Westbrook et al. 2020). Additional delays often occur when flower or seed production is reduced by external factors such as late frost, drought, insect attack, animal browse, storms, or blight infection.

When a wild-type diploid plant such as American chestnut is crossed with a compatible hemizygous (single transgene copy on one chromosome) transgenic relative, approximately 50% of progeny are expected to inherit and express the transgene. In practice, outdoor controlled pollinations using hemizygous transgenic pollen typically yield 35-45% transgene inheritance (Powell et al. 2020), potentially due to presence of airborne wild-type pollen during controlled pollination efforts. To generate pollen for controlled pollinations and accelerate chestnut reproduction, Darling pollen (and to a lesser extent mature Darling nuts) can be rapidly

80 produced indoors, which we here term “speed breeding”. As a plant breeding tool, speed
81 breeding is simply the manipulation of controlled, indoor environmental and horticultural
82 methods that accelerate the sexual maturity of plants. This process has been applied to various
83 row crops (Ghosh et al. 2018; Watson et al. 2018), and apple trees (*Malus* spp.) for breeding
84 (Flachowsky et al. 2011; van Nocker and Gardiner 2014).
85 Pioneering speed breeding work for American chestnut relied on production of male
86 catkins, which consistently form earlier than female flowers in both artificial (indoor) and natural
87 (outdoor) conditions. Male flowers can be induced in under one year when trees are fertilized
88 regularly and exposed to high intensity light with a prolonged photoperiod (Baier et al. 2012;
89 Klak et al. 2021). Typically, pollen produced indoors year-round has been collected, frozen, and
90 used for outcrossing to wild-type trees during the outdoor breeding season. Female flowers
91 have also been induced in under one year (Baier et al. 2012), but prior to the work presented
92 here, crosses to female flowers rarely reached maturity. In this work, we show that mature seed
93 production indoors is possible, in conjunction with plant care improvements such as repeated
94 repotting as seedlings grow, fertilization of trees under high light, and control of pests and
95 pathogens.
96 After outcrossing results in more distantly related individuals that have both inherited
97 *OxO*, resulting transgenic trees can be intercrossed. Crossing two hemizygous trees will
98 theoretically result in a pool of offspring that is approximately 25% non-transgenic, 50% single-
99 copy (inheriting *OxO* from one parent), and 25% two-copy (inheriting *OxO* from both parents).
100 Darling trees with two copies of *OxO* are considered homozygous for the transgene. Pollen from
101 trees homozygous for *OxO* would be ideal for producing Darling seeds from controlled
102 pollinations, since 100% of offspring from crosses with Darling homozygous pollen are expected
103 to inherit the *OxO* gene for blight tolerance. Trees homozygous for *OxO* are not a restoration
104 priority, but would be valuable for research and production of increasingly diverse blight-tolerant
105 trees, especially from outcrosses on mother trees that are declining from blight.
106 Chestnuts can also be initiated from various tissues and micropropagated using tissue
107 culture to contribute to the breeding program. Through tissue culture, we have produced flowers
108 on both clonally propagated plantlets, and flowers which mature into viable nuts on seedlings.
109 To protect limited and valuable chestnuts, collection of immature embryos (here termed “embryo
110 rescue” following Pilkey [2021]) and *in vitro* germination could help indoor-pollinated chestnuts
111 reach maturity. Embryos are a common explant for initiating various plant species in tissue
112 culture, including walnut (*Juglans* sp.) (Kaur et al. 2006), Japanese holly (*Ilex crenata*) (Yang et
113 al. 2015), and grape (*Vitis* sp.) (Jiao et al. 2018). Because of their juvenility, embryos from many
114 plant species are inclined to regenerate and multiply *in vitro* with relative ease (Kyte et al. 2013).
115 To our knowledge, embryo rescue has not been previously reported with American chestnut.
116 Additionally, molecular methods to identify homozygous transgenic Darling chestnut trees have
117 not been previously described.
118 The objectives of this study were to 1) describe production of indoor American chestnut
119 pollen and female flowers that are capable of producing fertile nuts, 2) test initiation timing of an
120 *in vitro* embryo rescue protocol for American chestnut, 3) test the *in vitro* embryo rescue
121 protocol with embryos produced through indoor pollinations and, 4) describe two molecular
122 methods and results for distinguishing homozygous from hemizygous trees, including a copy
123 number test using qPCR and a multiplex PCR genotyping assay.

# Methods

**Transgenic American chestnut speed breeding components**

Speed breeding methods were deployed to produce quantities of pollen and sexually mature chestnuts under controlled indoor conditions by eliminating seasonality for this deciduous species. We have observed early flowering under diverse indoor high-light conditions, but for enhanced development, our experience identified seven crucial components and parameters of chestnut speed breeding. Comparable material with equivalent specifications may be used based on the materials listed herein. (1) Full-spectrum grow lights (Mars Hydro SP 3000) on 15-16 hour photoperiods year-round, with PAR levels at the top seedling leaves near the outdoor June 21 noon maximum. (2) Acidic, porous potting mix (Premier Pro-Mix BK55), enhanced with micronutrients (Micromax G90505 1.3 mL/L), time-release granular fertilizer (Osmocote Plus 1.3 mL/L), and perlite for additional aeration. (3) Water-soluble fertilizers administered every two to three days, including a flowering nutrient (Roots Organic Terp Tea Bloom 4 mL/L) and micronutrients (alternating FloraFlex, Drip or Mills A&B [8 mL/L], with amendments of phosphorus, potassium [Drip flex 2.6 mL/L] and calcium [Drip CalMag 1.3 mL/L]). (4) Larger pot sizes as each plant grows, to confine but not fully restrict roots. Nuts with exposed radicles were sown in one- or two-gallon pots, and successively transplanted to 5–7 gallon pots and then to as large as 15 gallon pots before they became root-bound. (5) Growth room temperatures ideally in the range of 24°C daytime and 17°C at night, averaging approximately 20°C. (6) Relative humidity controlled to approximately 30-50%. (7) Timely identification and treatment of pest and pathogen outbreaks as part of an integrated pest management scheme, including treatments for fungus gnats, spider mites, and powdery mildew.

**Indoor production of pollen and fertile nuts on transgenic American chestnut seedlings**

We typically collect transgenic pollen from catkins directly onto glass microscope slides, which can be used immediately for pollinations or stored for freezing in air-tight slide fixing jars (e.g., Slide-Fix Caplugs, Evergreen Labware). Receptive female flowers were typically pollinated multiple times with the same pollen to maximize chances of pollination during optimal receptivity. Compared to outdoor breeding, the indoor setting with smaller trees allowed easier access to both female flowers and pollen and facilitated multiple pollinations. Additionally, indoor pollination was performed throughout the year, in contrast to the approximately two-week pollination window outdoors. After successful pollination, fruit development (bur growth) was observed and visibly mature burs were confirmed to contain fertile seeds.

We observed that lapses, such as high temperatures or low relative humidity, could cause the sensitive female flowers to abort. Despite this sensitivity, we observed more than half of all lab-generated female flowers reached maturity over a recent two-year period. Burs began to split and fully mature chestnuts were harvested indoors on average 13 weeks after the first pollination. Chestnuts were then stored for approximately two months at room temperature in sphagnum moss moistened with Bt (e.g. Summit Mosquito Bits, to suppress fungus gnats). For comparison, typical stratification recommendations for chestnuts specify approximately 2-3 months at 1-4.5C (Growing Chestnuts 2025). Nuts with radicles visible after this shortened stratification were then sown in two-gallon pots and gradually introduced to increasing amounts of fertilizer and intensity and day length under grow lights.

167
168 **Establishing an embryo rescue protocol for American chestnut**
169 Wild-type seeds were used to test the *in vitro* embryo rescue method. Two wild-type
170 flowering American chestnut trees in Sherburne, NY were selected to be pollinated. To prevent
171 open pollination and ensure accurate timing, immature pistillate flowers were bagged with
172 standard 3D pollination bags (PBS International, York, United Kingdom). When female flowers
173 became receptive, wild-type pollen was applied to flowers in 18 pollination bags on each tree.
174 One pollination bag on each tree was left unopened to serve as a non-pollinated control.
175 Pollination bags covered the flowers for the duration of the study to prevent open pollination and
176 provide protection. The pollination bags containing flowers were cut from the trees at intervals of
177 2 weeks, 4 weeks, 6 weeks, and 8 weeks after the controlled pollinations were conducted. Two
178 pollination bags were removed from each tree for a total of four pollination bags for extractions
179 every two weeks. At eight weeks the non-pollinated control bags were cut from both trees.
180 To sterilize the tissue prior to micropropagation, the immature chestnuts were first
181 excised from the involucre (developing bur). Chestnuts were sprayed with 70% ethanol for 30
182 seconds, then submerged and continuously shaken in a solution of 50% bleach (~3% sodium
183 hypochlorite) plus 0.01% Tween-20 for 15 minutes, followed by three, five-minute rinses with
184 sterile distilled water. To remove the seed coat, the seed was cut latitudinally at the hilum and a
185 longitudinal cut was made to open and peel away the pericarp. Once the seed coat was
186 removed, ovules or embryos were aseptically removed. Explants were considered an ovule if
187 the fertilized ovule could not be distinguished from its unfertilized neighbors. The explant type
188 was considered an embryo if the functional ovule (FO) that had been fertilized could be
189 identified. All observable ovules or embryos were extracted and placed with the micropyle facing
190 downward into a multiplication medium (full-strength Lloyd and McCown Woody Plant medium
191 salts (WPM), 109 mg/L Nitsch and Nitsch vitamins, 1 μM benzyladenine (BA), 0.01 μM indole-3-
192 butyric acid (IBA), 3% sucrose, 0.7% agar, with pH adjusted to 5.5). Explants were kept on a
193 light shelf receiving 31 μmol/m$^2$/s of light with a 16-hour photoperiod. All ovules and embryos
194 were transferred to fresh media every two weeks.
195 Explants were observed for germination every two weeks at the end of their transfer
196 cycle. In this experiment, germination was defined as the emergence of a shoot apical
197 meristem. All explants were given a value of “0”. When an embryo germinated, it was given a
198 value of “1”. Explants were also monitored for contamination and death. At the end of the
199 experiment, the overall germination rate and elapsed time until germination were observed.
200 Contamination rates for each treatment were calculated, however, explants that were
201 contaminated were removed from the germination rate calculation. Shoot maintenance
202 (including multiplication and elongation stages) in tissue culture, as well as *ex vitro* rooting and
203 potting protocols were performed using previously established methods for American chestnut
204 (Oakes et al., 2020).
205
206 **DNA isolation**
207 50-100 mg of fresh, young chestnut leaf tissue were manually disrupted in a 1.5 mL
208 Eppendorf tube and extracted using DNeasy Plant Pro kit (Qiagen 69204) using the standard
209 manufacturer protocol.
210

211 **Reconstruction of Darling 54 T-DNA insertion junctions**
212 Illumina reads from 16020, one of the T1 founder lines used for Darling chestnut
213 breeding were mapped to T-DNA using Geneious (11.1.5) program using the short read paired-
214 end setting. Next, paired reads spanning right border (RB) and left border (LB) were manually
215 extracted and assembled using SPAdes assembler (3.10.0) (Bankevich et al. 2012). Contigs
216 from RB and LB reads were both mapped to both T-DNA and Ellis v1.1 genome assembly
217 (Westbrook et al. 2025) using BLAST to determine T-DNA junction to the Ellis v1.1 Chr04.
218 Reconstruction of the intact, single copy T-DNA insertion in Darling 54 is presented in
219 Supplemental Figure 1.
220
221 **Real time quantitative PCR assays**
222 Quantitative PCR reactions used Bio-Rad iTaq Universal SYBR Green Supermix in 20µl
223 reactions including 1ng template DNA and 1µM final concentration of each primer. Each
224 biological sample was done in triplicate for each primer set (*OxO* as described below, with actin
225 and ubiquitin reference genes; see Supplemental Table 1 and Carlson et al. (2022)).
226 Amplification was carried out in a Bio-Rad CFX Connect qPCR cycler. The qPCR program
227 began with a three-minute denaturation step at 95°C, followed by 39 cycles of 95°C for 10s, and
228 60°C for 30s with reads taken at the end of each 60°C step. This was followed by a melt curve
229 (65°C to 95°C in 0.5°C increments) to check for single specific PCR products and specificity of
230 amplification. Copy number controls were Darling 58 (known single copy) and Darling 4 (known
231 two copy). Relative copy number values were analyzed via normalized expression (ΔΔCq) using
232 Bio-Rad CFX Maestro software.
233
234 **Genotyping assays**
235 *OxO* qPCR primers were described previously (Zhang et al. 2013) but were used in our
236 experiments for end-point PCR detection of the *OxO* T-DNA construct. The Darling 58 event
237 was described in the USDA-APHIS Petition for nonregulated status and supplemental
238 information (Powell et al. 2020). For Darling 54, RB and LB PCR primers were designed based
239 on reconstructed T-DNA junctions and multiplex PCR was designed based on the deletion on
240 Caden.04G062600. Primer sequences and expected PCR sizes are provided in Supplemental
241 Table 1. PCR was performed using 2x GoTaq Green Mastermix (Promega M7122).
242 Approximately two ng of DNA were used per 20 µL PCR reactions containing two µM of each
243 primer. All reactions were run with 40 cycles of 94°C for 30s, 55°C annealing for 30s and 68°C
244 extension for 60s. Final PCR products were run on a 1.5% TAE gel and imaged after ethidium
245 bromide staining.

246 **Homozygous transgenic plant development and pollen production**
247 Since American chestnuts are generally self-incompatible, and all transgenic chestnuts
248 of a given event are derived from a single founder genotype, genetic diversification requires
249 outcrossing transgenic individuals over multiple generations. Intercrosses between transgenic
250 lines were performed when both pollen and female flowers were available on T2, T3 or T4
251 transgenic trees that were not full siblings. Some immature seeds were subjected to embryo
252 rescue protocols to speed recovery of offspring and are represented by the TGA, TGG and TGJ
253 families in this report. For the ULL family from which we obtained fully mature burs indoors,

attention to each of the speed breeding parameters was crucial. In the case of the TGA family, Darling 54 T2 pollen was crossed to T2 individuals derived from different families to minimize relatedness. After female flowers were observed on indoor potted transgenic plants, transgenic pollen was applied as described above. As the fertilized burs developed, they were harvested via embryo rescue. Embryos were germinated *in vitro*, propagated, and advanced to shoot culture. Transgene homozygosity of the rescued embryo cultures was confirmed by both qPCR for copy number and T-DNA genotyping using leaf tissue. One of the confirmed homozygous embryo cultures (TGA002) was propagated, rooted, acclimatized, and maintained under the same high-light conditions described above until catkins developed. Pollen from TGA002 was first produced under high light in the winter of 2023/2024 and stored at -80ºC to preserve pollen viability.

**Outdoor controlled pollinations with homozygous transgenic pollen and early testing**

In the summer of 2024, 15 wild-type chestnut mother trees at four USDA-APHIS permitted field locations in New York state were selected for controlled pollinations using pollen homozygous for *OxO* (TGA002). In mid-June, immature pistillate flowers on the wild-type mother trees were bagged to prevent open pollination. Approximately two weeks later, homozygous pollen (TGA002) was applied to receptive stigmas and pollination bags were placed back over the developing flowers. On one of the mother trees (#1 in Table 1), pollen hemizygous for OxO (UET001) was also applied to additional female flowers as a control.

At eight weeks post-pollination, a portion of developing seeds were removed from four of the 15 mother trees (1 – 4 on Table 1) for early OxO inheritance testing. Several open-pollinated seeds from each mother tree were also collected to serve as wild-type controls. Immature seeds were peeled from the involucre. All fertilized seeds containing an embryo were peeled, and tissue from the embryo was sliced into segments and collected into microcentrifuge tubes. Embryos were tested for presence and activity of the OxO enzyme using a colorimetric histochemical assay regularly deployed on other chestnut tissues (Liang et al. 2001). All other TGA002 control-pollinated flowers from the 15 mother trees were harvested at maturity (~14-16 weeks post-pollination). Cores from the cotyledons of mature, viable seeds were tested for OxO inheritance and activity using the same histochemical assay.

# Results

**Indoor production of pollen and female flowers, bred to produce fertile nuts**

An overview of our speed breeding processes, including induced female and male flowers from transgenic American chestnuts, is shown in Figure 1. Owing to the shortened maturation time frame from speed breeding, pollen-bearing male catkins were observed as early as five months after sowing. Most seedlings produced pollen within one to two years after sowing. The emergence of bisexual catkins including female flowers ranged from seven months to more than two years after sowing. Intercrosses between two hemizygous transgenic seedlings can potentially give rise to the three genotypic outcomes indicated, including homozygous transgenic lines. On the bottom left of Figure 1, female flowers that were successfully fertilized are able to develop into full-sized burs that remain green, which can be harvested to obtain fully mature seeds in about 13 weeks. In conjunction with the modified

stratification described above, this process resulted in more rapid radicle emergence (as little as one month), and resulting seedlings could be transplanted directly in soil in about two months after harvest. These seedlings can then be subjected to the next speed breeding cycle, repeating the process and reducing the seed-to-seed time to just under two years.

Immature burs that are not fully expanded but remain attached for at least six weeks are also viable for speed breeding with the use of embryo rescue methods as shown on the bottom right of Figure 1. In some cases, burs aborted prematurely, but ovules from immature seeds were embryo-rescued, followed by *in vitro* germination and multiplication, which takes around five months. Acclimatization and rooting of the multiplied lines take around three months, but can yield many clonal plantlets at this stage. In our experience, rooted embryo rescue lines take 12-14 months to produce male flowers (pollen) under speed breeding conditions.

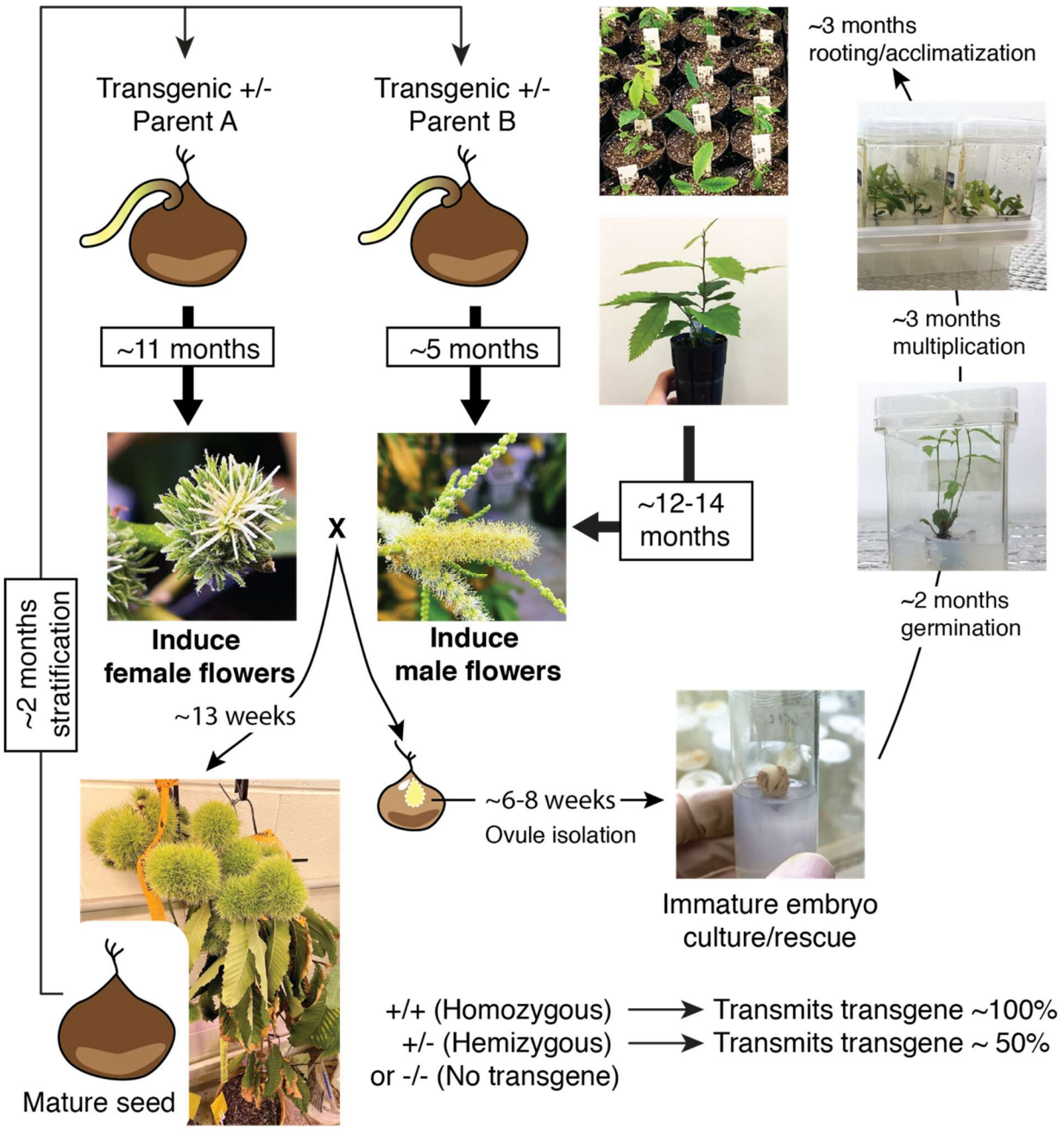


***Figure 1. Transgenic American chestnut speed breeding scheme.*** *Speed breeding of transgenic American chestnut can result in the induction of male flowers in as little as five months and female flowers in as little as 11 months after seed stratification. Some of the crosses performed result in fully matured burs (left downward arrow). Mature seeds can be harvested from fully matured burs and, after two months of stratification, seedlings can be cycled back for indoor flower production. Premature burs can be subject to immature embryo rescue via tissue culture (right downward arrow). Manually excised functional ovules were surface-sterilized, germinated and multiplied in vitro, and rooted plantlets on soil cycled back for indoor flower production. Genotyping of transgenic seedlings from either mature seeds or*

*embryo rescue can be performed to assess the copy number of the transgene of interest. Bolder arrows denote seedling or plantlet subject to speed breeding flower production, while lighter arrows denote timelines for each stage. Notations: -/- indicates no transgene, +/- indicates a hemizygous transgenic line, and +/+ indicates homozygous transgenic line.*

**Initiation timing of an *in vitro* embryo rescue protocol for American chestnut**

Embryo rescue tests on immature burs from two wild-type American chestnuts helped determine the age and conditions necessary for successful germination of dissected ovules. As ovules mature, they are called embryos; both terms are used here with a transition at approximately six weeks. A total of 100 chestnuts were removed from immature burs on involucres from controlled pollinated chestnuts at various time points (Figure 2). Between one and 15 healthy ovules were excised from each chestnut and put into growth medium. In total, 363 healthy ovules or embryos were excised from controlled pollinated chestnuts (2-weeks: 18 chestnuts [n=131 ovules]; 4-weeks: 29 chestnuts [n=117 ovules]; 6-weeks: 40 chestnuts [n=93 ovules or embryos]; 8-weeks: 18 chestnuts [n=17 embryos]). Significant differences between extraction time treatments were observed in mean germination rate ($F=11.26$, $p<0.001$) and contamination rate ($F=10.66$, $p<0.001$) (Figure 2A). The mean germination rate of ovules or embryos extracted from each treatment was: 0% at two weeks, 2% at four weeks, 8% at six weeks, and 23% at eight weeks. Contamination rates in treatment were: 0% at two weeks, 5% at four weeks, 14% at six weeks, and 45% at eight weeks. The development and differentiation of ovules into zygotic embryos can be observed by eight weeks post-pollination (Figure 2B). Average time elapsed between embryo rescue and *in vitro* germination was 133 days for four-week, 114 days for the six-week, and 70 days for the eight-week treatment. Embryos from the eight-week time point germinated significantly faster than four- and six-week time points ($F=3.982$, $p=0.004$).

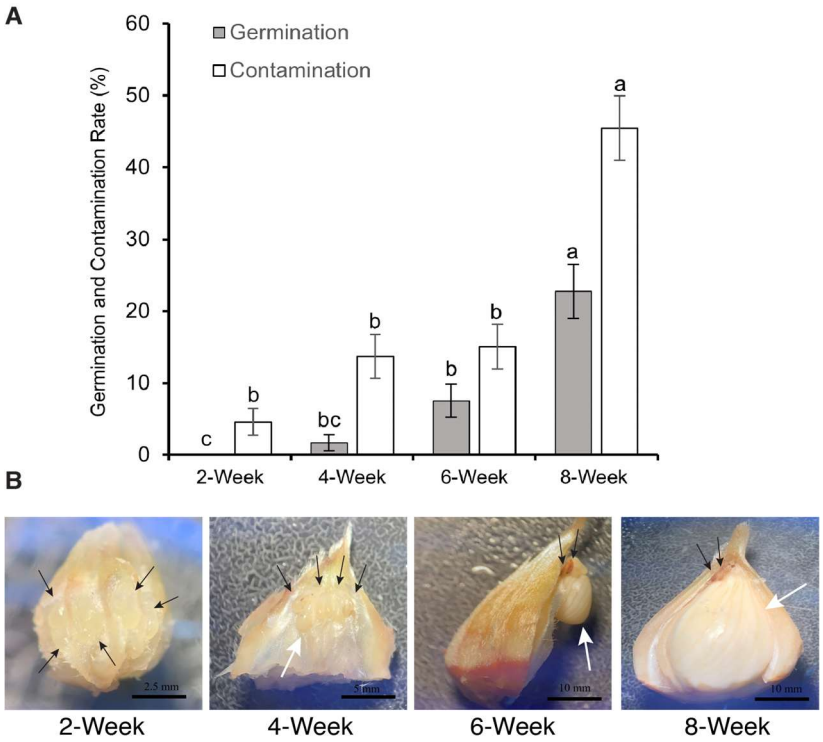


***Figure 2. Timing of embryo rescue in American chestnut immature embryos.*** *(A) Germination and contamination rate from American chestnut ovules collected 2-, 4-, 6- and 8-weeks after pollination. Error bars indicate +/- one standard error of the mean. (B) Photos of corresponding stages dissected from each week. In each inset image, black arrows point to immature ovules and white arrows point to functional ovules.*

**Test of the *in vitro* embryo rescue protocol with embryos produced through indoor pollinations**

Indoor controlled pollinations between receptive female and pollen-producing Darling American chestnut lines (T2, T3, and T4) led to a total 33 burs, of which 18 were harvested while immature and 15 matured on the plant. From the 18 immature burs (six to eight weeks old), 44 ovules were dissected and subjected to embryo rescue, resulting in 23 embryo rescue lines that germinated *in vitro* and were tested for *OxO* copy number. These lines ranged from multiple generations and represented by several families (Supplemental Table 2). From the 15 fully mature burs, 37 fertile nuts were obtained, germinated and grown in soil (the ULL family). In the speed breeding setting, we obtained on average, 2.5 full-term, viable nuts per bur, which was similar to the success rate for mature chestnuts from repeated controlled pollination from outdoor settings.

**Two molecular methods and results for distinguishing homozygous from hemizygous trees**

Initial experiments determined that transgene copy number (and therefore zygosity) can successfully be determined on plantlets derived from embryo rescues using real time quantitative PCR (qPCR). The bar plot in Figure 3A based on qPCR data indicates two copies of *OxO* in Darling 4, a known two-copy T0 transformant (Newhouse et al. 2014), zero copies in

the Ellis wild type, and one copy in SX58 (T0 of Darling 58) as expected. This method also correctly identified a homozygous Darling line TGA002 with two *OxO* gene copies, and two hemizygous Darling lines, TGG001 and TGJ001, that show a single copy of the *OxO* gene (Figure 3A). However, this qPCR assay only targets a portion of the *OxO* cDNA, and does not take the transgene insertion location into account. Based on the T-DNA insertion sites of Darling 54 and 58, we designed a multiplex genotyping assay to test for the endogenous insert position and differentiate D54 versus D58 transgene location. All Darling material currently used for pollen production is derived from the D54 T0 line, SX54, though some was previously misidentified as D58 (Steiner et al. 2017). In total, three homozygous D54 lines were identified from 24 lines derived from embryo rescue. From fully mature burs, one out of the 37 fertile nuts that we genotyped was homozygous for D54. All three genotypic outcomes (non-transgenic, hemizygous, and homozygous) were present in seedlings from embryo rescued as well as fertile nuts. In conclusion, we successfully recovered homozygous D54 transgenic lines from both fully matured and immature burs using speed breeding (Supplemental Table 3).

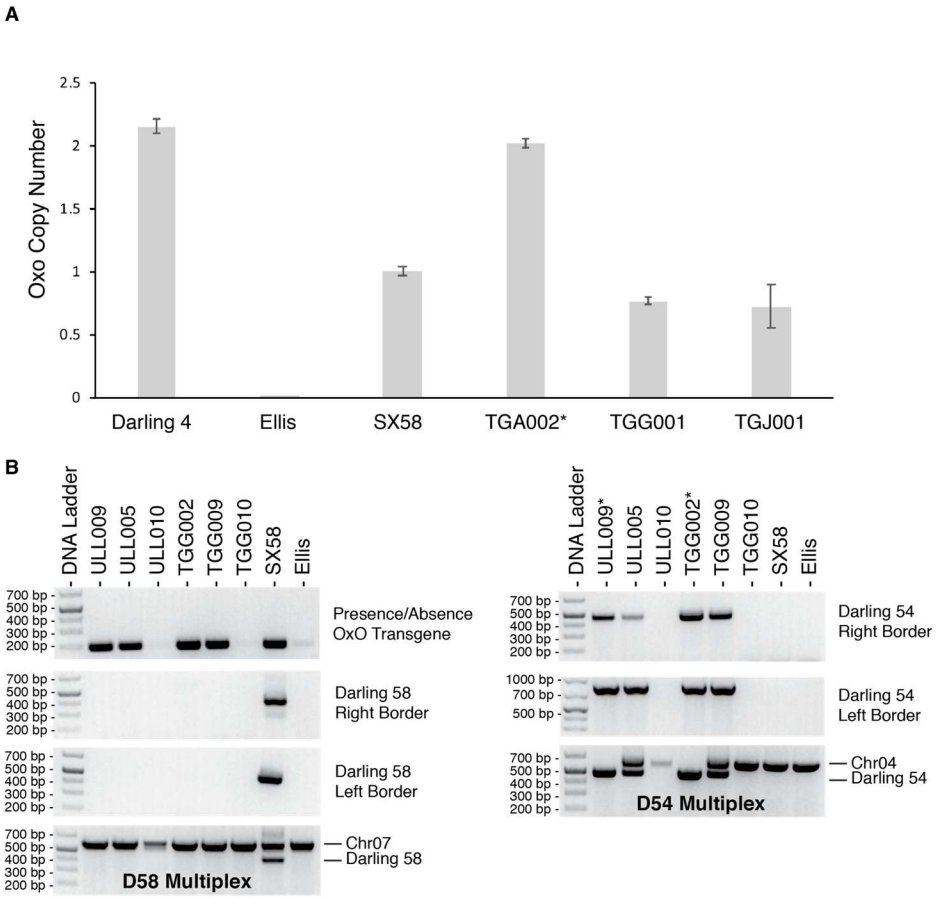


***Figure 3. Genotyping methodologies for Darling 54 and Darling 58 from embryo rescues and seedlings from mature nuts. (A)*** *Bar plots of the relative OxO copy number from quantitative PCR, showing two-copy T-DNA present in T0 Darling 4 and absence/zero-copy of OxO-containing T-DNA in the wildtype Ellis line. T0 Darling 58 (SX58) shows a single-copy state, T3 TGA002 shows two-copy state (*indicates a potentially homozygous line based on qPCR), T5 TGG001 and T5 TGJ001 show single-copy states. (**B**) Genotyping assay for OxO,*

*Darling 58 (D58) and Darling 54 (D54) on two T5 transgenic families (ULL and TGG), T0 Darling 58 (SX58) and the Darling wild type progenitor (Ellis). The OxO qPCR primer set determines the presence or absence of the OxO transgene regardless of insert location. D58 RB and LB determine presence of the OxO T-DNA on Chr07, while D58 Multiplex provides amplification of the insertion site on Chr07 and D58 LB. Similarly, D54 RB and LB determine presence of OxO T-DNA on Chr04, while D54 Multiplex provides amplification of the insertion site on Chr04 and D54 RB. Based on these assays, ULL and TGG T5 families are derived from D54. ULL009 and TGG002 are homozygous for D54 while ULL005 and TGG009 are hemizygous for D54. ULL010 and TGG010 are wild-type null segregants.*

**Outdoor pollinations with homozygous pollen**

Outdoor controlled pollinations with homozygous Darling 54 pollen yielded a total of 578 seeds in 2024, of which 132 were harvested for early testing and 446 were harvested at maturity. Concurrent pollinations with hemizygous pollen on one of the same mother trees yielded 47 seeds harvested early and 224 harvested at maturity. Of the early harvests from a homozygous pollen parent, all but one seed (131/132) tested positive for *OxO*, while mature harvests showed 417 *OxO*-positive out of 446 total nuts, for a combined total of 95% *OxO* inheritance from a homozygous parent (Table 1). In contrast, pollinations with hemizygous pollen resulted in a combined total of 41% inheritance (119 out of 271 total harvested nuts), which is typical based on our extensive experience with transgenic pollinations.

***Table 1. Inheritance rates of the OxO transgene in offspring of controlled crosses with both hemizygous and homozygous pollen.*** *Testing time (early vs. mature) is shown in weeks after pollination; Mother Tree numbers refer to separate individual trees. Mother tree 4* is a Japanese/European hybrid 'Bouche de Betizac'; all others are fully American to the best of our knowledge.*

| | | Early testing (8 weeks) | | Mature seed testing (~14 weeks) | | |
|---|---|---|---|---|---|---|
| **Pollen type** | **Mother Tree** | **n Positives / n Tested** | **OxO Inheritance Rate** | **n Positives / n Tested** | **OxO Inheritance Rate** | **Mean OxO Inheritance Rate** |
| **Hemizygous (UET002)** | 1 | 17/47 | 0.36 | 102/224 | 0.46 | **0.41 (n=271)** |
| **Homozygous (TGA002)** | 1 | 41/41 | 1 | 35/35 | 1 | **0.95 (n=578)** |
| | 2 | 15/15 | 1 | 17/22 | 0.77 | |
| | 3 | 12/13 | 0.92 | 3/3 | 1 | |
| | 4* | 63/63 | 1 | 22/30 | 0.73 | |
| | 5 | | | 54/54 | 1 | |
| | 6 | | | 24/26 | 0.92 | |
| | 7 | | | 125/131 | 0.95 | |
| | 8 | | | 6/6 | 1 | |
| | 9 | | | 7/7 | 1 | |
| | 10 | | | 1/1 | 1 | |
| | 11 | | | 2/2 | 1 | |
| | 12 | | | 5/5 | 1 | |
| | 13 | | | 69/74 | 0.93 | |
| | 14 | | | 47/50 | 0.94 | |

## Discussion

This paper reports on three novel contributions to American chestnut restoration that also have potential applicability to other *Castanea* species and hybrids as well as to other tree species. First, we've bred fully mature transgenic chestnuts indoors. Most previously described speed breeding methods are aimed at rapidly advancing generations for domesticated crop species (Ghosh et al. 2018; Watson et al. 2018; Bhatta et al. 2021). By adapting speed breeding techniques for trees, we show that American chestnuts can be induced to produce both pollen and viable female flowers, bypassing the temporal constraints of the annual outdoor growing season, and in a much-reduced maturation timeline compared to outdoor breeding. In turn, male- and female-mature chestnut plants can be strategically and successfully crossed indoors to advance chestnut generations. These contribute to overcoming the founder effect of a single transgenic event and to diversifying the genetic pool for potential restoration plantings (Newhouse and Powell 2021; Westbrook et al. 2021). Our methods to speed breed American chestnut can be replicated in almost any lab or growth room using cost-effective, space-advantageous and widely available wide-spectrum LED lights provided that the right cultural conditions are met. The parameters may also have to be refined at different locations, depending on the environmental controls available in each speed breeding lab. We also showed that fully developed mature burs containing fertile nuts can also be produced and harvested under speed breeding conditions. Our initial yield of 2.5 nuts per bur is consistent with yields from outdoor controlled crosses with transgenic pollen. Stratification can be shortened to around two months (until they grow a substantial radicle) before sowing in soil. After a few weeks of acclimatization, newly sprouted seedlings that survive can be genotyped, and cycled back into the speed breeding process.

Second, the embryo rescue methods and results reported in this paper are novel to American chestnut. The most suitable time point to extract functional ovules from American chestnut for embryo rescue is at six to eight weeks after pollination, though some germination was observed as early as four weeks. Dissected functional ovules typically take two to four weeks to germinate. Multiplication and rooting of germinated embryos can also result in rapid production of whole plants that can be used for speed breeding or other field studies. This also allows the dissection of seeds from immature burs from speed breeding that would otherwise have aborted early, providing a way to use this intentionally or as emergency rescue of important crosses. Embryo rescue feeds back into the speed breeding cycle (Figure 1), by making it possible to produce pollen from a plant that is homozygous, or a tree that performs well in the field, without the delay associated with traditional breeding to produce mature nuts. Another advantage of working with immature burs for embryo rescue is that the embryos can be cloned easily (Xing et al. 1999; McGuigan et al. 2020), which allows for larger population increases in a shorter time for genotypes of interest. However, seedlings transferred from tissue culture to soil may take longer to flower compared to seedlings from mature seed (Figure 1). Beyond speed breeding or indoor pollinations, embryo rescue can be employed to recover whole plants from field-pollinated seeds that have been impacted by disease or environmental damage, or would otherwise not be able to mature on the tree. For example, we have employed

embryo rescue to recover important transgenic progeny from a wild-type hybrid mother tree that flowered outside of the typical pollination season. We performed controlled pollinations at the end of August, and embryo rescue in late September. Developing ovules would have typically been killed by the onset of cold temperatures, but embryo rescue allowed successful production of healthy plants from this late-season cross.

Third, combined methods of speed breeding and genetic analysis were aimed at identifying lab-grown homozygous transgenic American chestnut for pollen production to be crossed with wild-type mother trees in forests and germplasm conservation orchards located across the Eastern United States (Westbrook et al. 2020). These methods led to the production of the first homozygous D54 transgenic line, which produced viable pollen and yielded 95% transgene inheritance in offspring from several mother trees. Pollen homozygous for OxO is a useful tool for research and breeding but is not seen as a critical component for restoration. Production and testing of additional Darling 54 homozygous lines, derived both from tissue culture and from full-term indoor breeding, is underway to better understand crossing and inheritance rates with higher sample sizes from additional genetic backgrounds. These methods can be easily adapted to produce and identify homozygous offspring of other transgenic events in the future.

In addition, this work highlights the value of integrating the methods of speed breeding, embryo rescue, and event-specific genotyping assays. We have validated two genotyping assays to determine copy number and to distinguish different transgenic lines. The qPCR assay provides a method to determine copy number on any transgenic events containing the *OxO* transgene, regardless of the promoter driving *OxO* or *a priori* knowledge of the genomic location for the transgenic event. This method can be helpful in early screening and characterization of transgenic lines, including improved lines such as those with wound-inducible promoters driving *OxO* expression (Carlson et al. 2022). On the other hand, performing the multiplex D58 and D54 assay on breeding material routinely can provide assurance to the speed breeding process, while also providing copy-number status.

The speed breeding and embryo rescue methods reported in this study are not restricted to transgenic chestnuts, although this method is especially important for controlled field pollinations under USDA-APHIS confined field trial permit conditions. Recent genomic advancements, such as the availability of high-quality genome assemblies of multiple *Castanea* species, facilitate research on transgenic American and traditional hybrid breeding work (Westbrook et al. 2025). Chestnut speed breeding as a methodology is relevant to all breeding-associated gains, such as trait development, genomic selection, backcross breeding, and genetic diversification efforts. There is also interest in exploring the use of similar methods to reduce generational time for other plants such as citrus (SIPS 2021). In addition, our embryo rescue methods can be utilized for conservation of unique genotypes, especially from threatened trees. It may also be useful to accelerate breeding of other *Castanea* species for agricultural purposes such as F1 *C. sativa* x *dentata* and *C. pumila* x *dentata* hybrids (Klak et al. 2021). This work highlights the continual improvements that can be made to rapid breeding of American chestnut and to rescuing immature embryos, both of which are critical for American chestnut research as we work towards the restoration of this iconic species back to its native range.

**Contributions and Acknowledgements**

TK, HP, AN and EHT wrote the manuscript, and conceptualized the experimental design for speed breeding and molecular assays. TK, VGM, and HP performed speed breeding experiments and performed crosses to produce immature and mature seed. HP developed and tested embryo rescue protocols. TK, VGM, HP, and DM performed crosses to produce immature seeds, contributed to embryo rescue experiments, and provided DNA samples. DM performed and analyzed quantitative PCR. AO and EHT performed T1 mapping and junction analysis for Darling 54. All authors reviewed the manuscript and provided edits.

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**Supplemental Data**

Supplemental Figure 1: Darling 54 transgene insertion site on Chr04 of Ellis

Supplemental Figure 2: Images of mature and developing burs on sped bred American chestnut and histochemical assay from progeny from homozygous Darling 54 pollen

Supplemental Table 1: Oligo sequence and PCR product details

Supplemental Table 2: Darling 54 embryo rescue and mature chestnut seed lines and details

Supplemental Table 3: Segregation ratios of progeny from two heterozygous Darling 54 parents from embryo cultures and mature seeds

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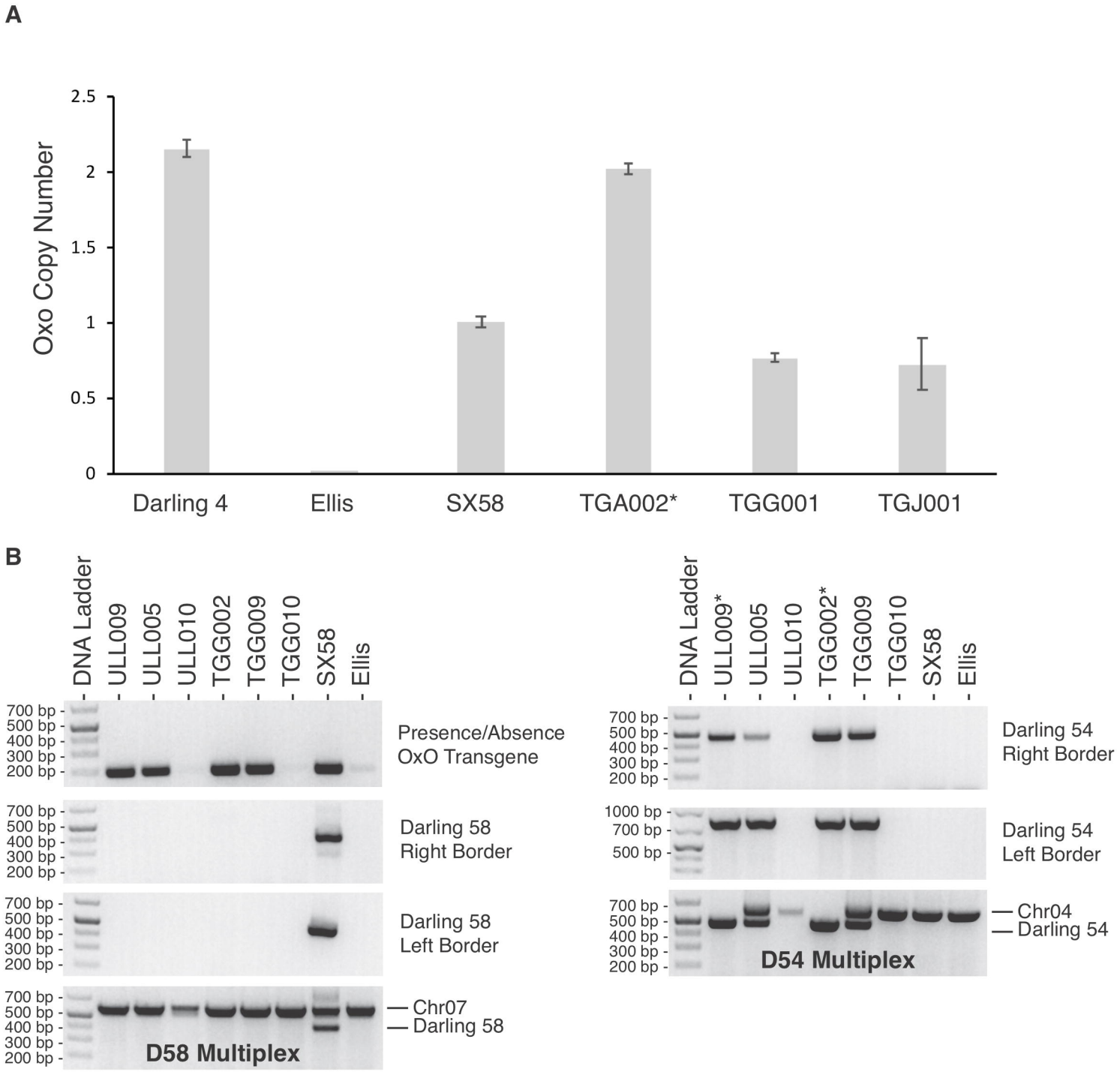
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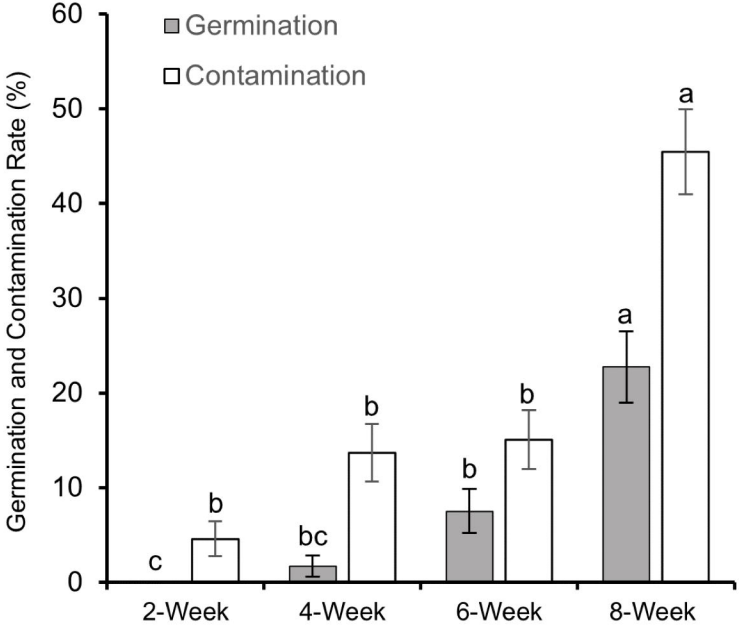


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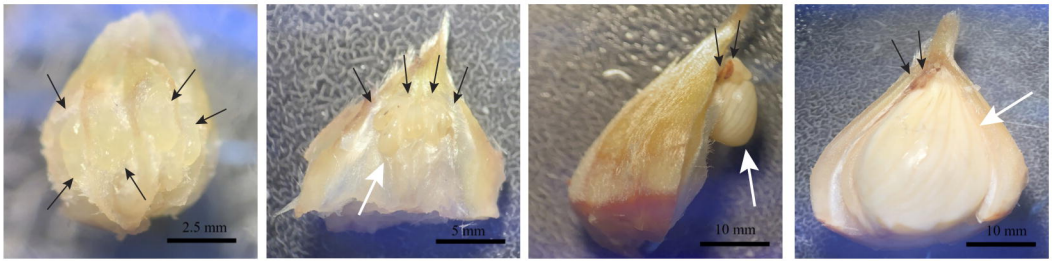


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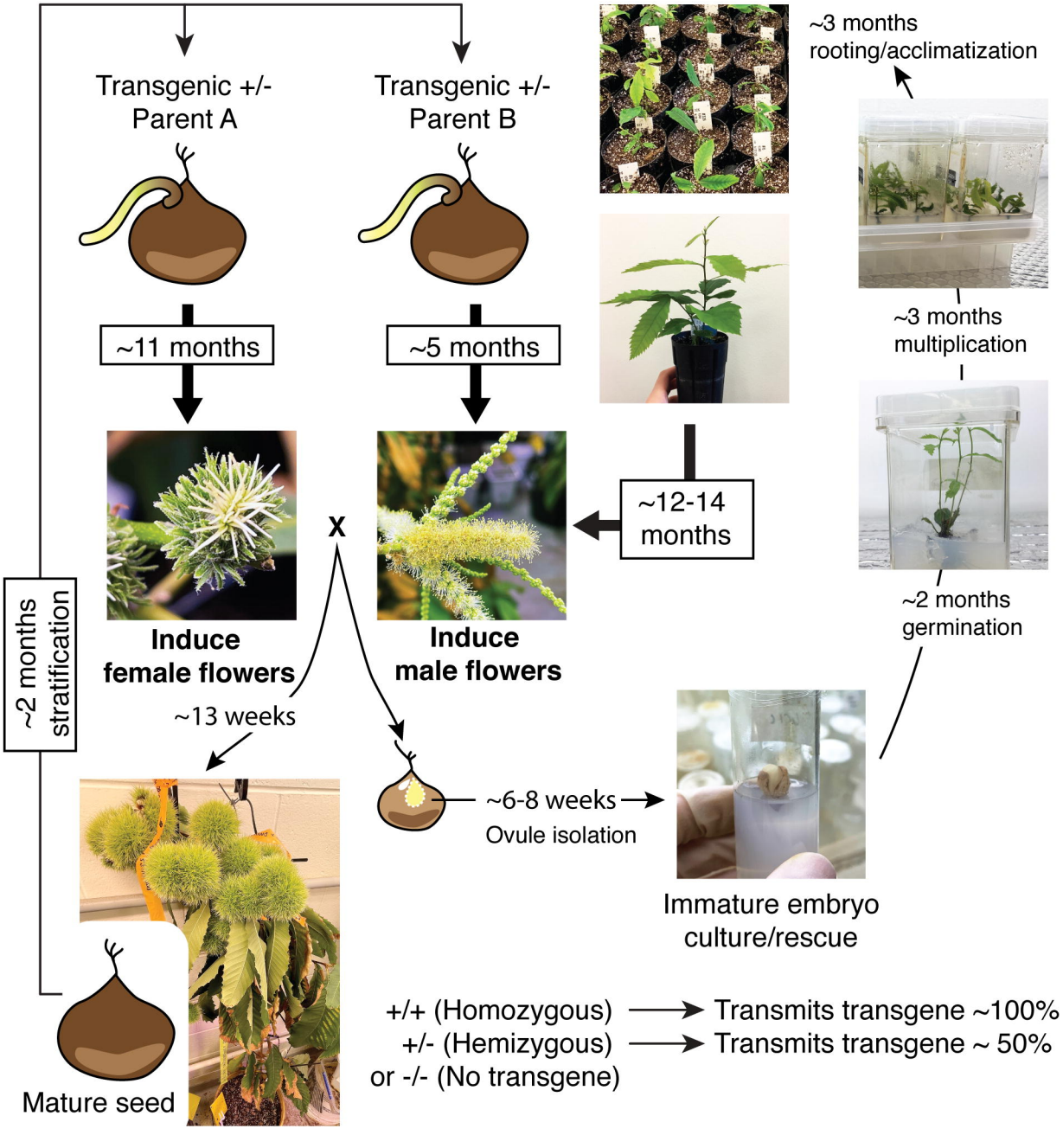
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+/+ (Homozygous) → Transmits transgene ~100%
+/- (Hemizygous) → Transmits transgene ~ 50%
or -/- (No transgene)