Title: Development of a transgene-free gene editing system in European Pear

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ABSTRACT

Gene editing has emerged as a useful tool for introducing valuable changes into germplasm that affect important traits and would otherwise require mutagenesis and many rounds of breeding. However, delivery of gene-editing machinery into plant cells has been limited in many horticultural species, including European pears, by needing to use agrobacterium-mediated transformation of leaf material. This system has rather low efficiency and requires the gene-editing machinery to be integrated into the plant's DNA, which then needs to bred back out as part of the regulatory process. Several tools have been developed in other crops that address some of these limitations. For example, the development of protoplast transformation systems, zygotic embryogenic culture, and somatic embryogenic culture, all explained in detail below. These systems, once developed for a crop species, improve the ability to transform plants without having to rely on agrobacterium. We proposed to apply these methods to European pear, such that as genetic targets and markers are developed for dwarfing, fire blight resistance, iron chlorosis, Armillaria resistance, and other desirable traits, we will have a system in place to edit the genes involved in these processes in existing and new rootstock cultivars. Here we describe successful plant regeneration from both leaf discs and seed tissue, initial successes in generating protoplasts from regenerated tissue, and work towards developing the "proof of concept" gene editing system for the purpose of demonstrating future successful transformation.

OBJECTIVES

Long-term objective: To establish a system for gene editing in pear, to allow the future development of germplasm with dwarfing, fire blight resistance, and other desirable traits.

Objective 1: Develop methods for generating pear protoplasts from in vitro micro shoots.

Objective 2: Develop zygotic embryogenic cultures in pear.

Objective 3: Develop somatic embryogenic cultures in pear

PROCEDURE

Plant Materials: Bartlett tissue, both *in vitro* cultures and immature fruit for seeds, were sent from the Waite lab to the Brown lab for use in regeneration protocols. *In vitro* cultures of OHxF 87 and 97 were also sent for future testing. For micropropagation at USDA, shoots were sub-cultured in Magenta GA-7 boxes (Magenta Corp., Chicago, IL, USA) with 40 ml medium per container. For Bartlett, the base medium used was PM2 (Pear Medium 2) which is similar to MS (Murashige & Skoog) but contains 2x of all mesos (Ca, Mg, P minerals), as well as 2.5 mg/L thiamine, 250 mg/L inositol, 30 g/L sucrose, 4.4 µM N6-benzyladenine (BA), 0.6% agar (A111, PhytoTechnology Labs, Shawnee Mission, KS, USA) adjusted to pH 5.7 and autoclaved. For OHxF97, the base medium used was Pear Rootstock (PRS-propagation) medium, which is similar to PM2 but contains 2.5x MS level of mesos (Ca, Mg, P minerals). OHxF87 was also grown on PRS-propagation medium, but with 1.2x of MgSO4 (instead of 2.5x).

Shoots were transferred into fresh medium every four weeks and multiplied. Pear shoot cultures were grown at 20°C under a 16-h photoperiod with an average of 50 µM/m2s irradiance.
Immature fruit was collected from Bartlett trees grown at the Columbia View experimental orchard facility co-managed by USDA and Washington State University. Trees received standard pruning and management. In vitro shoots of Bartlett pear obtained from the Waite Lab were maintained in the Brown Lab on Murashige and Skoog medium (MS)(Murashige and Skoog, 1962) modified with 5 uM 6-benzylaminopurine (BAP), 0.5 uM indole-3-butyric acid potassium salt (K-IBA), 3% w/v sucrose, and 0.6% w/v A111 agar (Phytotech Labs) with pH adjusted to 5.7 before autoclaving. Cultures were kept under a 16-hr photoperiod with transfer every 3 weeks.

Protoplast isolation from callus (Obj. 1): Protoplast isolation trials were performed on leaf sections cultured in the dark for 3 weeks on Adventitious Shoot Medium (ASM): our basal pear MS medium supplemented with 100 mg/L myo-inositol, 3% w/v sucrose, and 0.6% w/v A111 Agar adjusted to pH 5.7 supplemented with hormones as described in Kotoda et al. (2006) 15 uM Thidiazuron (TDZ), 5 uM NAA. Callused leaf sections were placed in an enzyme digestion solution for 24 hours, filtered, washed, and then floated on a dextran gradient before harvest and encapsulation in alginate beads. Successfully beaded protoplasts were suspended in an agar-free, liquid ASM amended with 0.6M mannitol to prevent cell rupture during culture.

Embryogenesis from seeds (Obj. 2): Immature 'Bartlett' fruit were sent from the Waite lab to the Brown lab in late July for embryogenesis attempts. Seeds were fully mature but seed coats had not yet hardened. Fruit were surface disinfested with NaDCC and refrigerated in sterile containers for three days while media was prepared. Fruit were cut transversely and extracted seeds were cultured with seed coats intact in Petri plates containing Embryo Induction Medium (EIM), a modified Murashige and Skoog (MS) medium containing 5uM BAP, 30 uM a-naphthaleneacetic acid (NAA), 3% w/v sucrose, and 0.6% w/v A111 Agar (James et al, 1984). Medium pH was adjusted to 5.7 before autoclaving. Cultures were placed in the dark and checked daily for evidence of contamination. After 3 days seed coats were removed, seeds were cut into quarters, placed on fresh EIM, and cultured further in the dark. After four weeks, seed sections were transferred to an Embryo Development Medium (EDM): MS supplemented with 5 uM 6-BAP, no auxin, 3% w/v sucrose, 0.6% w/v A111, at pH 5.7. Half of the plates were placed under 16-hour light, half in the dark, and were observed weekly.

Shoot organogenesis from leaves and petioles (Obj. 3): Leaves and petioles of 'Bartlett' taken from in vitro shoot cultures were used for shoot organogenesis trials. Using fresh #10 scalpels, leaf margins were removed and the remaining leaf tissue was sectioned into approximately 5 mm by 10 mm rectangular pieces (Fig 1). These were placed abaxial side down in Petri dishes containing Adventitious Shoot Medium (ASM): MS medium supplemented with 100 mg/L myo-inositol, 15 uM Thidiazuron (TDZ), 5 uM NAA, 3% w/v sucrose, and 0.6% w/v A111 Agar adjusted to 5.7. Petiole tissue was given fresh cuts

at both the proximal and distal ends to ensure no nodal tissue was present and also placed on ASM. Cultures of both tissues were kept in the dark and observed weekly.

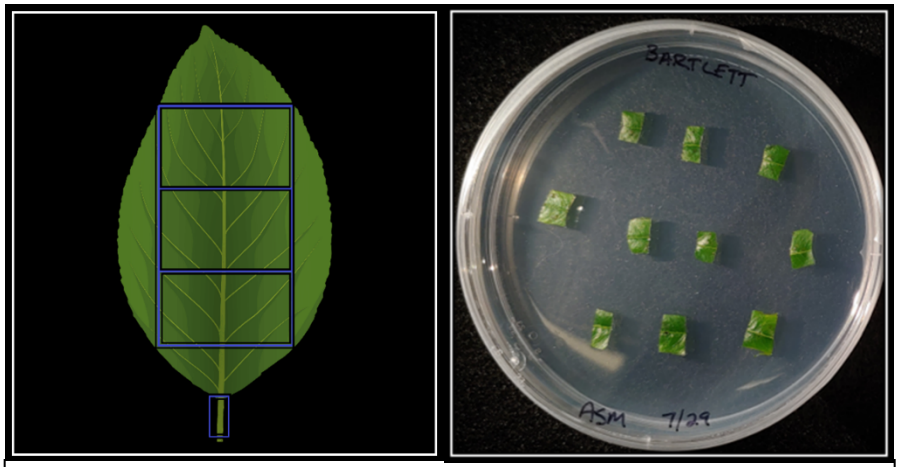

Figure 1: A. Cutting design for leaf sections to be cultured. B. Freshly cut leaf sections at day 0 of culture.

Development of transformation vectors: Vectors from the Wu lab (cite) were ordered for the purpose of building gene-editing vectors to target *phytoene desaturase (PDS)* in pears. Target sequences within *PDS* will be determined using CRISPOR (crispor.tefor.net), which determines off-target sites. Primers will be designed to insert the *PDS* targets into the vectors for delivery into plant cells.

RESULTS AND DISCUSSION

Results

Protoplast isolation from callus

Protoplast isolation trials were performed and we were able to isolate (Fig 2) and encapsulate small numbers of protoplasts in calcium alginate beads for further development. We have not yet been able to induce cell division in the isolated protoplasts using our current media formulations, and will focus on determining best media for this in the coming year. We also noted that in contrast to apple, callus obtained from the Bartlett leaf sections proved to be mildly hydrophobic, preventing good contact when immersed in the enzyme solution used to digest cell walls. Reducing the surface tension of the enzyme digestion medium may improve contact and future formula optimizations of the Shoot Organogenesis Medium could produce a callus source more amenable to enzyme digestion similar to what we have seen in other crops. Once protoplast isolation efficiencies are improved, a multifactorial study of plant growth regulator influences on cell division can begin.

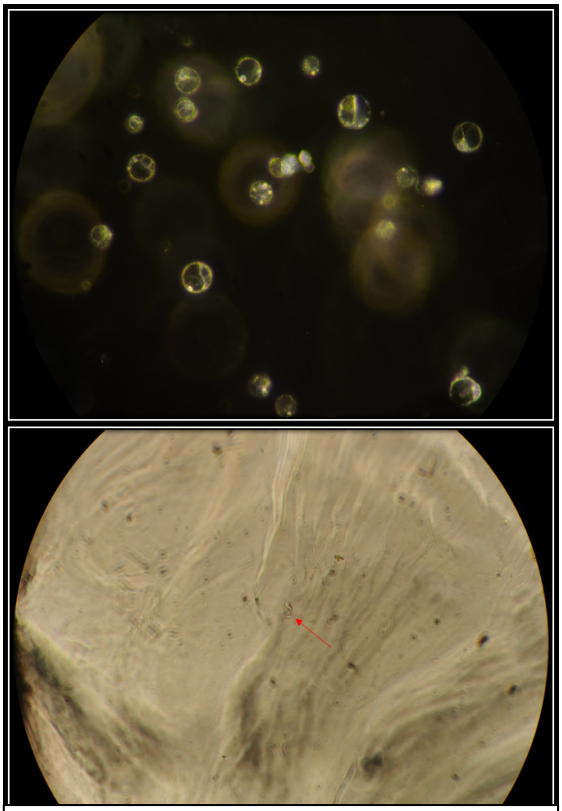

Figure 2. Top, protoplast isolated from Bartlett leaf organogenesis. Bottom, Bartlett protoplast in alginate indicated by

Embryogenesis from seeds

Our initial efforts produced several structures that look promising. Wounded surfaces of cotyledons exhibited obvious callus after only two weeks on Embryo Induction Medium (EIM) and roots emerged from some

of the callus tissue. Adventitious root formation made identification of adventitious shoot or embryo structures difficult. Despite the difficulty in discerning adventitious shoot or embryo tissue (Fig 3), we believe both tissue types could be used for a future transformation protocol. We retained cultures of three of these and will attempt to multiply them as embryos and/or generate shoots to establish a complete regeneration system suitable for gene editing. Future work to improve embryogenesis efficiency and embryo development could include seed introductions at different developmental stages, testing additional media salt formulations, and use of alternative PGR treatments.

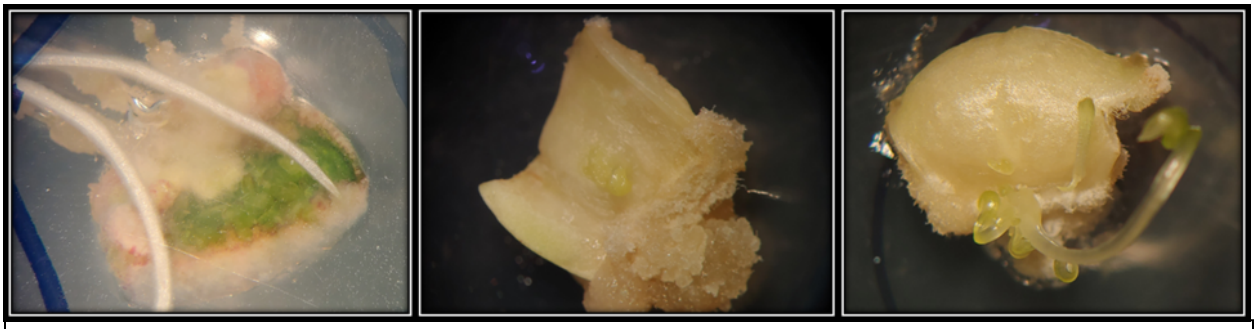

Figure 3. Cultured seed pieces with developing shoot structures, perhaps embryos.

Shoot Organogenesis from Bartlett leaves and petioles

Nearly all leaf sections produced callus, initiating primarily at the proximal end and particularly near vascular tissues such as leaf veins and midribs (Fig 4). In this first trial, the conversion rate of callus into adventitious shoots was low, with 0% of petioles and less than 10% of leaf pieces producing shoots. Work to convert the obtained adventitious shoots into stable multiplying shoot cultures is still in progress. A media salt and vitamin optimization study is ongoing but has not yet generated data. Once data from this experiment is obtained, we can move forward with testing various plant growth regulator combinations to optimize the system and improve efficiency.

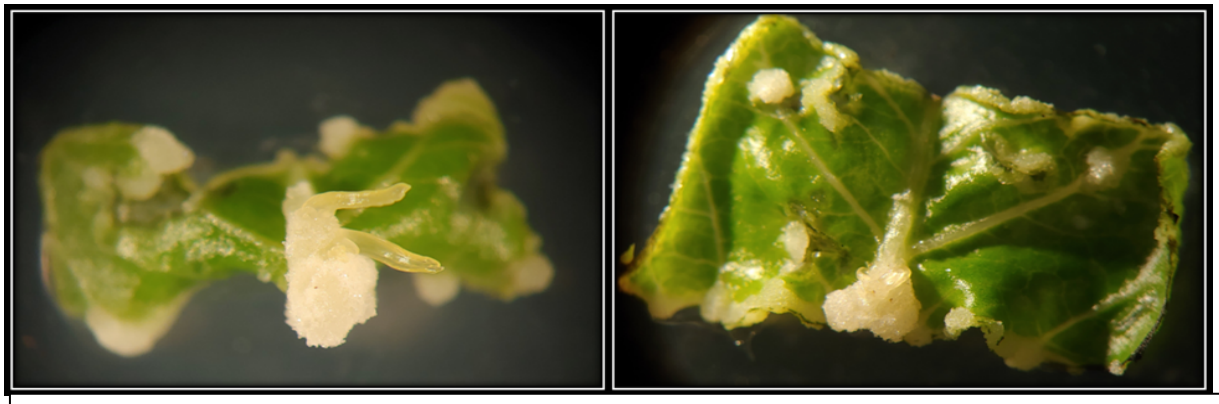

Figure 4. Cultured leaf pieces exhibiting somatic organogenesis.

Transformation vectors and gene editing targets

To demonstrate that transformation is working correctly and that the gene editing system is functional, it is common practice to initially target a gene whose mutation will cause an immediate visual effect. Similar to other groups using gene editing in tree crops and other plant species, we identified *phytoene desaturase (PDS)* as an initial target. A recent study from the Wu lab (Ming et al 2022), tested different aspects of CRISPR systems to determine the best parts to use for pear calli. We ordered the parts to build our own CRISPR system in the coming year using Golden Gate cloning.