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, including development of protoplast transformation systems and somatic embryogenic culture, 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 propose to apply these methods to European pear, such that as genetic targets and markers are identified for dwarfing, disease, and pest resistance, we will have a system in place to edit the genes involved in these processes in existing and new rootstock cultivars. For each of these traits, specific genes have not yet been identified, however ongoing work in rootstock breeding programs, the USDA germplasm repositories, and other labs are currently narrowing down the genetic regions important for dwarfing and fire blight. Successful development of these systems has the potential to reduce the risks of adopting new scion or rootstock varieties for growers, by introducing precise advantageous edits/mutations into established varieties (eg: Bartlett, OHxF 87 and 97). In the past year, we have worked towards optimizing tissue regeneration in Bartlett and OHxF 97, as well as testing enzyme combinations for protoplast isolations.

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: Optimize shoot tissue regeneration from leaf discs of OHxF87 and 97.

Objective 2: Optimize methods for isolating and culturing pear protoplasts from in vitro micro shoots.

Objective 3: Design and generate gene-editing machinery.

PROCEDURE

Plant Materials:

In vitro shoots of Bartlett pear were maintained in the Brown Lab on Murashige and Skoog (MS) media modified with 5 µM BAP, 0.5 µM indole-3-butyric acid potassium salt (K-IBA), 3% w/v sucrose, and 0.6% w/v A111 agar with pH adjusted to 5.7 before autoclaving. Cultures were kept under a 16-hr photoperiod with transfer every 3 weeks.
For micropropagation in the Waite Lab, Bartlett shoots were sub-cultured in Magenta GA-7 boxes (Magenta Corp., Chicago, IL, USA) with 50 ml medium per container. For Bartlett, the base medium used was PM2 (Pear Medium 2) with the addition of 1 mg/mL Gamborgs vitamin solution (G219, PhytoTechnology Labs), or Murashige and Skoog (MS) media containing Gamborgs vitamins (M404, PhytoTechnology Labs). PM2 is similar to MS (Murashige and Skoog, 1962) but contains 2x of all mesos (Ca, Mg, P minerals), as well as 2.5 mg/L thiamine, 250 mg/L myo-inositol. Both medias contained 3% w/v sucrose, 5 µM meta-Topolin (mT), 0.6% agar (A111, PhytoTechnology Labs), and were adjusted to pH 5.7 and autoclaved. For OHxF97, the basal 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). Shoots were transferred into fresh medium every four weeks and multiplied. Pear shoot cultures were grown at 22°C under a 16-h photoperiod with an average of 50 µmol/m2s irradiance.

Optimizing tissue regeneration (Obj. 1)

Shoot organogenesis experiments to test and optimize the effects of etiolation (a period of dark growth) and rooting media on regeneration followed a similar protocol in both labs: Bartlett and OHxF 97 plantlets were trimmed and transferred to either a multiplication or rooting media and placed in the dark for 1 week. At the beginning of week 2, all plants were removed from dark and places in the light for an additional week. At the beginning of week 3, half of the plants grown on rooting media were placed back in the dark for 2 weeks. All other treatments remained in light for the same duration. At the end of week 4, shoot regeneration protocols were performed: leaf discs were cut or punched from the midrib region of young, expanding leaves from each treatment and placed on regeneration media. For Bartlett, full leaves were also excised and wounded by stabbing the leaves with forceps. Regeneration media consisted of full-strength MS media (for Bartlett) or NN69 media (for OHxF 97, (Nitsch and Nitsch, 1969)) with 15uM TDZ, and 5uM NAA. OHxF 97 leaves were also pre-soaked in liquid media prior to placement on plates to avoid oxidative browning, which has been an issue in the past for this cultivar. Bartlett plates contained 9-12 leaf discs each, each plate was considered one replicate, with 3 replicate plates per treatment, and two full runs of the experiment separated by a week. OHxF 97 plates contained between 15-25 leaf discs, with 3 replicate plates per treatment. Plates were kept in darkness for 3 weeks and moved to light. Both total shoots

and numbers of discs with regenerating shoots were counted at 4, 5, and 6 weeks and are reported below. A Student's t-test was used to determine significant differences between the means of different treatments. Media comparisons can be found in Table 1. Treatment comparisons can be found in Table 2.

Table 1. Media for Etiolation and Rooting experiment, prior to leaf excision:

	Multiplication Media	Rooting Media
MS media containing Gamborg's Vitamins (M404)	4.44g (1x)	2.22g (0.5x)
Hormones	5uM BAP, 0.5uM K-IBA	5uM K-IBA
Sucrose	30g (3%)	15g (1.5%)
Agar (A111)	6g	6g
pH	5.5	5.8

Table 2. Treatments compared in etiolation and rooting experiment

Media prior to leaf excision	Dark treatment (W1-W2-W3-W4)	Cut method
Multiplication Media	Dark-Light-Light-Light	Leaf disc/square
Multiplication Media	D-L-D-D (OHxF 97 only)	Leaf disc/square
Rooting media	D-L-L-L	Leaf disc/square
Rooting media	D-L-L-L	Full leaf, random stabs (Bartlett only)
Rooting media	D-L-D-D	Leaf disc/square
Rooting media	D-L-D-D	Full leaf, random stabs (Bartlett only)

Optimizing plant protoplast isolation and culturing (Obj. 2)

In the Waite Lab, protoplast isolations were attempted, closely following the pear protocol from Ochatt and Power (Ochatt and Power, 1988), and building on previous year's findings. Briefly, 0.3-0.5g of recently unfurled, fully expanded leaves from Bartlett *in vitro* plantlets were harvested into CPW 13M media (recipe in Table 3), cut into 1-2mm strips, and soaked for 1 hour to plasmolyse the cells. During this hour, the enzyme solution was made fresh by adding 1.0% Cellulase Onozuka RS, 0.1% Pectolyase Y-23, 5mM 2-(N-morpholino) ethanesulfonic acid (MES) solution, and 1.0% Polyvinyl Pyrrolidone (PVP) to 20mL CPW 13M media. Leaf strips were then transferred into dishes containing the enzyme solution and shaken at root temperature (25C) in very dim light at 40rpm. Digestions were carried out for 16 hours and 18 hours. Tissues were then run through a nylon sieve to remove cellular debris, and centrifuged at 100xg for 10 minutes. Protoplasts at the meniscus were then resuspended into 21% sucrose, re-centrifuged at 100xg for another 10 minutes, and observed on the microscope.

Table 3. Media for protoplast isolations:

CPW 13M	mg/L
KNO3 (Potassium nitrate)	101
KH2PO4 (Potassium phosphate)	27.2
CaCl2.2H2O (Calcium chloride dihydrate)	1480
MgSO4.7H2O (Magnesium sulfate heptahydrate)	246
KI (Potassium iodide)	.16
CuSO4.5H2O (Cupric sulfate pentahydrate)	.02
Mannitol	130g

Design of gene editing machinery (Obj. 3)

In previous years, we identified the *PHYTOENE DESATURASE (PDS)* gene as an initial target for gene editing, as alterations of this gene leads to visibly bleached tissues. To determine the guide RNA sequences that will be needed to target the CRISPR-Cas9 protein to the correct locations in the genome, we first identified all copies of the PDS gene, as well as homologous genes, in the Bartlett genome using the BLAST tool (Genome Database for Rosaceae, BLAST+ tool (Jung *et al.*, 2019)), and used the program JBrowse to determine that the genes were correct and expressed. We next used the CRISPOR program (https://crispor.gi.ucsc.edu/) to determine sequences within these genes that would be recognizable by the CRISPR-Cas9 enzyme. CRISPOR also determines all potential off-target sites, and suggests primers to use to test these sites for edits. Table 5 contains guide RNA sequences and numbers of potentially off-target sites.

RESULTS AND DISCUSSION

Tissue Regeneration

An important step in developing a protoplast-based gene-editing systems is developing efficient and robust regeneration protocols for common U.S.-grown pear genotypes that can be applied to any callus tissues that develop from protoplasts in future steps. In the previous year, we identified multiple parameters that increased regeneration rates in *in vitro*-grown Bartlett plantlets in preliminary experiments, including specific hormone combinations in the regeneration media, growing plantlets on rooting media prior to excising leaves for regeneration, and subjecting plantlets to a period of darkness prior to excising leaves. This year, we aimed to further test the darkness and rooting treatments, both to optimize these treatments for Bartlett, and test them with OHxF 97 plantlets. We

planned to test these also with OHxF 87, however a contamination event this year drastically reduced our numbers of OHxF 87 plantlets.

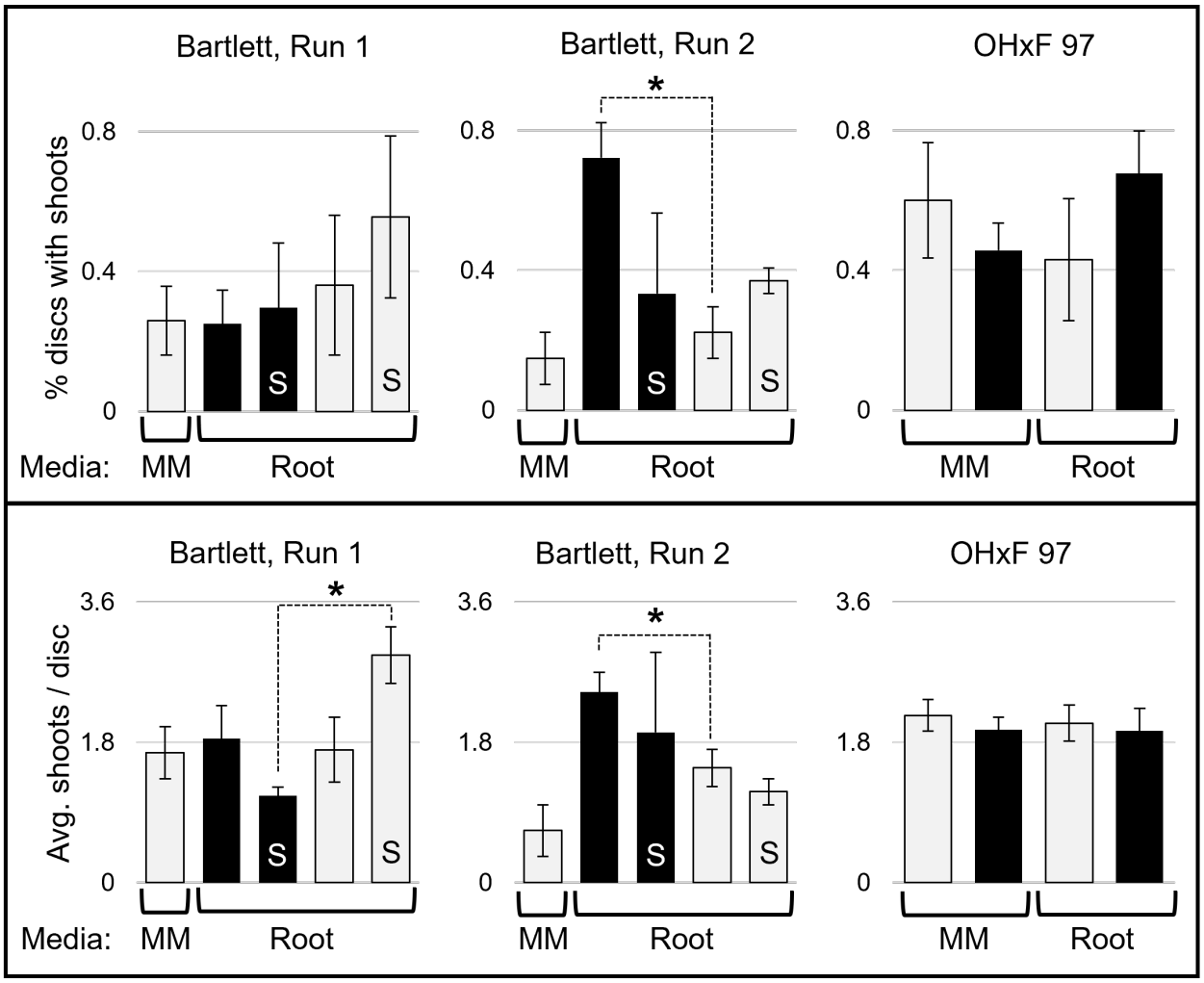

Figure 1. Regeneration efficiencies and shoot organogenesis averages at week 6 in leaf material from plantlets grown in different media and light conditions. A. Regeneration efficiencies, measured as the percent of leaf discs with at least one regeneration shoot, and B. the total number of regenerated shoots divided by the number of regenerating leaf discs are reported for Bartlett and OHxF 97. A fully replicated experiment was run twice for Bartlett leaves, spaced one week apart. Prior to leaf excision and placement on regeneration media, in vitro plantlets were grown on either multiplication (MM) or rooting (Root) media, and subjected to either an extended dark period or kept in light. Light gray bars represent light treatment, and black bars represent dark treatment. "S" represents leaves that were wounded via stabbing with forceps. Student's t-tests were used to determine significant differences in two-way comparisons of the means of regeneration results from plants grown on different medias, in different light conditions, or using different cut methods. Asterisks indicate significance levels of $p<0.05$.

At weeks 4, 5, and 6 after leaves and leaf discs were placed on regeneration media, we observed and calculated regeneration efficiencies, measured as the percentage of discs

that had at least one regenerating shoot (Figure 1A, week 6 reported), as well as average shoots per disc, measured as the total number of regenerating shoots divided by the number of discs with at least one regenerated shoot (Figure 1B, week 6 reported). Regeneration on the multiplication media used in this experiment ranged from 14.8% to 25.9% (Figure 1A), which shows a marked improvement from 3% last year when the same base media was used. Statistical comparisons were made between different medias (MM vs. Root) for the same light conditions and cut methods, as well as different light conditions (DLLL vs DLDD) for the same media and cut methods, and few significant differences were found. In the first experimental run for Bartlett, no significant differences were found in regeneration efficiencies. However, in excised leaves that were wounded via stabbing with forceps, growth in darkness resulted in fewer shoots per leaf, but this effect was not seen in the second run of the experiment. In the second Bartlett run, a significant improvement could be seen in regeneration efficiency for plant material that had received the dark treatment, compared to light (Figure 1A, middle graph). Further, in the same run, a large increase in regeneration efficiency could be seen when both dark treatment and rooting media were used, as compared to multiplication media in the light (Figure 1A, middle graph). However, these differences were not seen in the first run (Figure 1A, left graph). This suggests a potential role for an unknown parameter that differed between the two runs. In past years, we have seen regeneration rates from OHxF 87 and OHxF 97 reach ~35%. While we did not see significant differences between rooting media or darkness treatments for OHxF 97 in this experiment, we calculated regeneration efficiencies between 43% and 67.7%, which shows improvement and suggests that this combination of media and hormones is beneficial for regeneration in this cultivar. Overall, we cannot conclude that the addition of rooting media and/or a dark treatment can improve regeneration rates alone, and we will continue to test these parameters together with other inputs. Further, we saw several improvements to regeneration rates with the methods used in this study over results from previous years.

Protoplast Isolation

One of the most common methods used for DNA-free gene-editing in woody plant species is polyethylene glycol (PEG)-mediated transformation of protoplasts, followed by regeneration of protoplasts into in vitro shoots that carry the gene-edit. Before testing transformation, we've been working towards developing a reliable protocol for isolating and culturing protoplasts from common U.S.-grown genotypes. In previous years, the Brown lab was able to isolate protoplasts using a modified protocol from similar experiments in grapes (Tricoli, 2019). This year in the Waite lab, protoplast isolations followed a similar protocol, with modification of enzyme concentrations and using the media outlined in previous experiments with pears (Ochatt and Powers, 1988). In contrast to experiments from last year, we did not find any tissue browning this year and thus began experiments without the addition of antioxidants, however future trials will test antioxidant addition. Our initial attempts this year results largely in incompletely digested cell walls. We also found that after collecting the digested tissues and filtering out the cells, protoplasts could be found in both the meniscus (top layer of the solution) and the bottom of the tubes after centrifugation, possibly due to that incomplete digestion. We

next planned to test three additional enzyme mixtures outlined in Table 4, both for 16h and 18h duration, however, backorder and delivery delays have hindered progress. We will continue to test these in the coming year.

Table 4. Enzyme mixtures to be tested for cell wall digestion and protoplast isolation

Protocol publication	Media base	Maceroenzyme R-10	Onozuka Cellulase R-10	Hemicellulase	Pectolyase Y-23
(Revilla *et al.*, 1987)	CPW 13M	-	1.0%	1.0%	0.1%
(Ochatt and Power, 1988)	CPW 13M	-	1.0%	-	0.1%
(Ochatt *et al.*, 1992)	CPW 13M	0.2%	1.0%	1.0%	0.1%

Gene editing machinery

In previous years, we determined that the use of Ribonucleoproteins (RNPs), a complex of pre-formed gene editing enzymes and guide RNAs, would be ideal for delivery of the gene editing machinery into plant cells via protoplast transformation. This year, we determined the sequences that would need to be purchased, once we develop a reliable protoplast regeneration system and are ready to test transformations. We searched the genome for the correct sequences of the PHYTOENE DESATURASE (PDS) gene that we identified previously as a strong initial proof-of-concept target, as editing of this gene results in bleached tissues. Next, we used the CRISPOR program to determine ideal guide RNA sequences that will be used to guide the editing machinery to the precise location in the gene. Upon entering a desired sequence (in our case, the first two exons of the PDS gene) into the program, CRISPOR scans the sequence and the rest of the Bartlett genome to identify sequences that are likely to be high efficiency and have low chances of editing off-target sites. Table 5 contains the target gene ID, as well as the guide RNA sequences that will be included to guide the gene editing enzymes to the correct locations. The table also includes the number of predicted off-target sites, which are sequences elsewhere in the genome that share some similarity with the target. The number of mismatches in these potential off-target site correlates with the likelihood of being edited, such that 3-4 mismatches is less likely than 1-2 mismatches. Once edited, these sites will be sequenced to select for plants in which no off-target sites have been edited.

Table 5. Guide RNA sequences for targeting pear PDS gene for gene-editing

Gene description	Gene ID	Possible guide RNA sequences	# of potential off-target sites
Phytoene Desaturase	04g02050 - Exon 1	TTGGCAGCTCAAGTTAGCAGCGG	4 (w/ 3 mismatches) 11 (w/ 4 mismatches)

		AAAGAAAAGGCATCGCATCGGGG	2 (w/ 3 mismatches) 22 (w/ 4 mismatches)
		AAGCTGTTTATAGAAGGCCCAGG	1 (w/ 3 mismatches) 6 (w/ 4 mismatches
Phytoene Desaturase	04g02050 - Exon 2	GTACTGTCAAGGTCTGGTCTTGG	7 (w/ 4 mismatches)
		TTAGCAGTACTGTCAAGGTCTGG	2 (w/ 3 mismatches) 5 (w/ 4 mismatches)
		TTTAACGGCTTGGTTGGGCGAGG	17 (w/ 4 mismatches)

REFERENCES

Jung S, Lee T, Cheng CH, Buble K, Zheng P, Yu J, Humann J, Ficklin SP, Gasic K, Scott K, Frank M, Ru S, Hough H, Evans K, Peace C, Olmstead M, DeVetter LW, McFerson J, Coe M, Wegrzyn JL, Staton ME, Abbott AG, Main D. 2019. 15 years of GDR: New data and functionality in the Genome Database for Rosaceae. Nucleic Acids Res **47**, D1137-D1145.

Murashige T, Skoog F. 1962. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarum **15**, 473-497.

Nitsch JP, Nitsch C. 1969. Haploid plants from pollen grains. Science **163**, 85-87.

Ochatt SJ, Chevreau E, Gallet M. 1992. Organogenesis from 'Passe Crassane' and 'Old Home' pear (Pyrus communis L.) protoplasts and isoenzymatic trueness-to-type of the regenerated plants. Theoretical and Applied Genetics **83**, 1013-1018.

Ochatt SJ, Power JB. 1988. Plant-Regeneration from Mesophyll Protoplasts of Williams Bon Chretien (Syn Bartlett) Pear (Pyrus-Communis L). Plant Cell Reports **7**, 587-589.

Revilla MA, Ochatt SJ, Doughty S, Power JB. 1987. A General Strategy for the Isolation of Mesophyll Protoplasts from Deciduous Fruit and Nut Tree Species. Plant Science **50**, 133-137.

Tricoli D. 2019. Renewal Progress Report for CDFA Agreement Number# 18-0397.