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

Report Type: Final Project Report

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Cooperators:

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Budget 1
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Item	2023		
Salaries			
Benefits			
Wages	$6,658.00		
Benefits	$509.00		
RCA Room Rental			
Shipping			
Supplies	$1,500.00		
Travel			
Plot Fees			
Miscellaneous			
Total	$8,667.00		

Footnotes:

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 'OHxF 87' and 'OHxF 97'.
Objective 2: Optimize methods for isolating and culturing pear protoplasts from in vitro micro shoots.
Objective 3: Design and generate gene-editing machinery.

Significant Findings

Growth of 'Bartlett' and 'OHxF 97' on rooting media or addition of a dark/etiolation period prior to leaf excision do not strongly influence adventitious shoot regeneration alone. However, regeneration rates improved overall from previous years. These findings suggest that our current base protocol has been further optimized, but that the addition rooting media and dark periods likely need to be combined with other inputs to increase shoot regeneration. These are important findings for the development of tissue culture-based propagation, both in research labs and the nursery industry.

Published pear protoplast isolation protocols led to overdigestion of tissue. This tells us that these protocols need refinement and updating, as they are likely missing information on variables that were not focused on in the publications (i.e. environmental factors, micropropagation media and conditions). Improvement of these protocols is an important step to obtain the protoplast cells needed to perform gene-editing without introducing transgenes, which will aid in the improvement of rootstocks.

Target CRISPR sequences within the Phytoene Desaturase (PDS) target gene were identified. PDS is an important target gene for testing whether a gene editing system is functioning properly, and identification of these target sequences means that we will have everything ready for editing once protoplast isolation, culturing, and adventitious shoot regeneration protocols are optimized and ready.

Results

Objective 1: Optimize shoot tissue regeneration from leaf discs of 'OHxF 87' and 'OHxF 97'.

Two major bottlenecks in developing and using transgene-free gene-editing systems are: 1) delivery of DNA or RNA into plant cells without use of Agrobacterium and without permanent DNA incorporation into the plant genome and; 2) regeneration of tissue, and then plants, from those cells. One way to achieve transgene-free, or DNA-independent, editing is to use protoplasts. This method has been developed in other woody crops [1, 2]. Protoplasts are plant cells with their cell walls removed, and thus can take up DNA or RNA directly and temporarily without relying on Agrobacterium. However, to generate protoplasts and optimize the process (described in Obj. 2), and to eventually recover plants from protoplasts, true-to-type callus tissue is needed, as well as a system to regenerate single cells into plants. In this objective, we focused on optimizing adventitious shoot regeneration in 'OHxF 97' and 'Bartlett'. We planned to optimize regeneration in 'OHxF 87' as well, however a contamination event this year drastically reduced our numbers of 'OHxF 87' plantlets.

In the previous year, we identified multiple parameters that increased regeneration rates in *in vitro*-grown Bartlett plantlets in preliminary experiments: 1. specific hormone combinations in the regeneration media; 2. growing plantlets on rooting media prior to excising leaves for regeneration; 3. subjecting plantlets to a period of darkness (etiolation) prior to excising leaves. This year, we aimed to further test and optimize these treatments.

Briefly, our protocols for these tests were as follows: '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', [3]) 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)

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

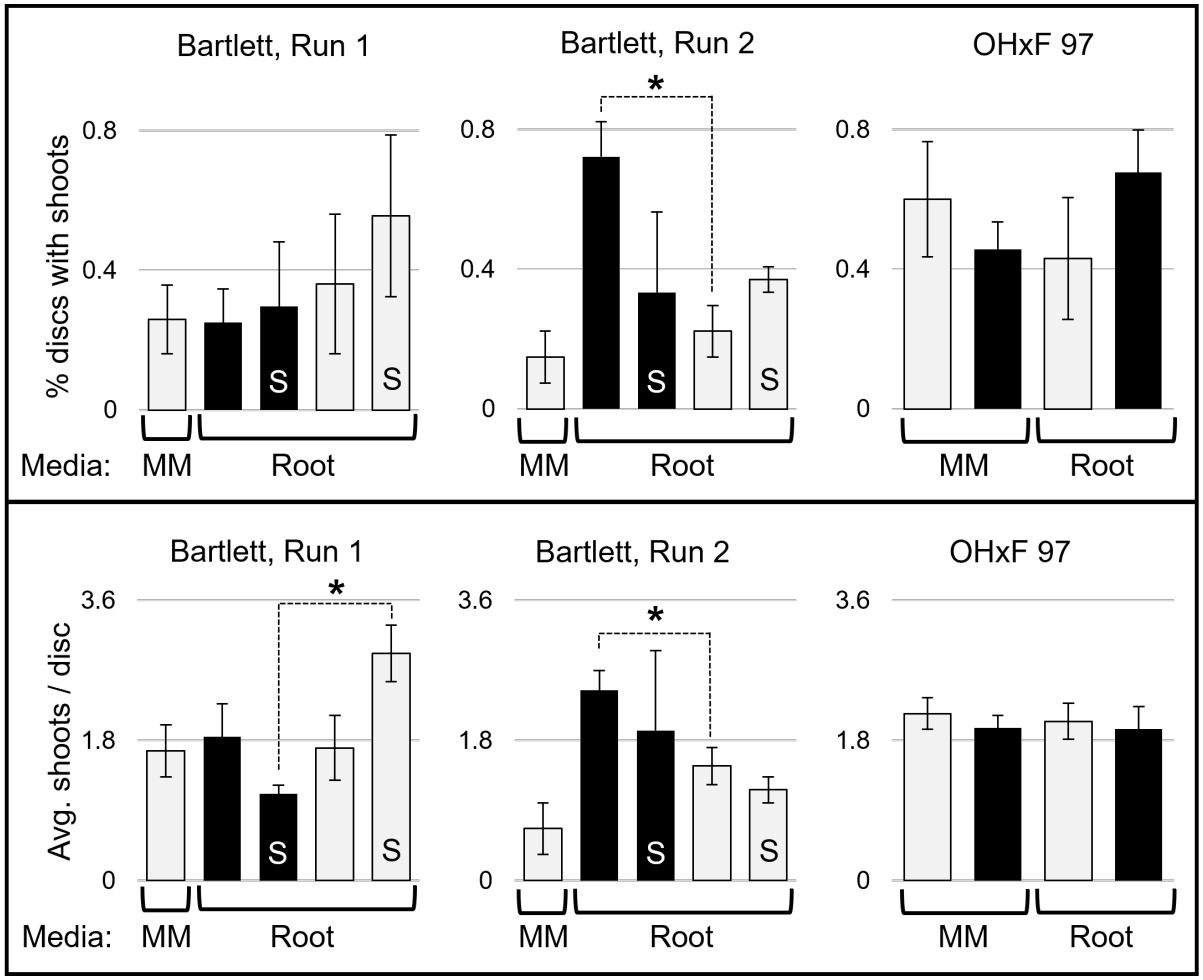

Figure 1. Regeneration efficiencies and shoot organogenesis averages at week 6 in leaf material from plantlets grown in different media and light conditions. A. Regeneration efficiencies, measured as the percent of leaf discs with at least one regeneration shoot, and B. the total number of regenerated 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 $p<0.05$.

(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.

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

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 edited gene. 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 [4]. 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 [5]. 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

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

for another 10 minutes, and observed on the microscope. Building on experiments from last year, we also added an antioxidant mixture to the digestion solutions.
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 tested three additional enzyme mixtures from published pear protocols, outlined in Table 4, both for 16h and 18h duration. We found that these enzyme mixtures and digestions times resulted in overdigestion and leaf material showed a high level of oxidative browning, despite adding antioxidants to the digestion solution (Fig. 2). No characteristic band of protoplasts could be seen in the filtered solutions, in contrast to the previous year (Fig. 2). In future trials, we will test different levels of antioxidants, varying concentrations of enzymes, and varying digestions times with these mixtures.

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

Protocol publication	Media	Maceroenzyme R-10	Onozuka Cellulase R-10	Hemicellulase	Pectolyase Y-23
Revilla et al., 1987 [6]	CPW 13M	-	1.0%	1.0%	0.1%
Ochatt and Powers, 1988 [5]	CPW 13M	-	1.0%	-	0.1%
Ochatt and Powers, 1992 [7]	CPW 13M	0.2%	1.0%	1.0%	0.1%

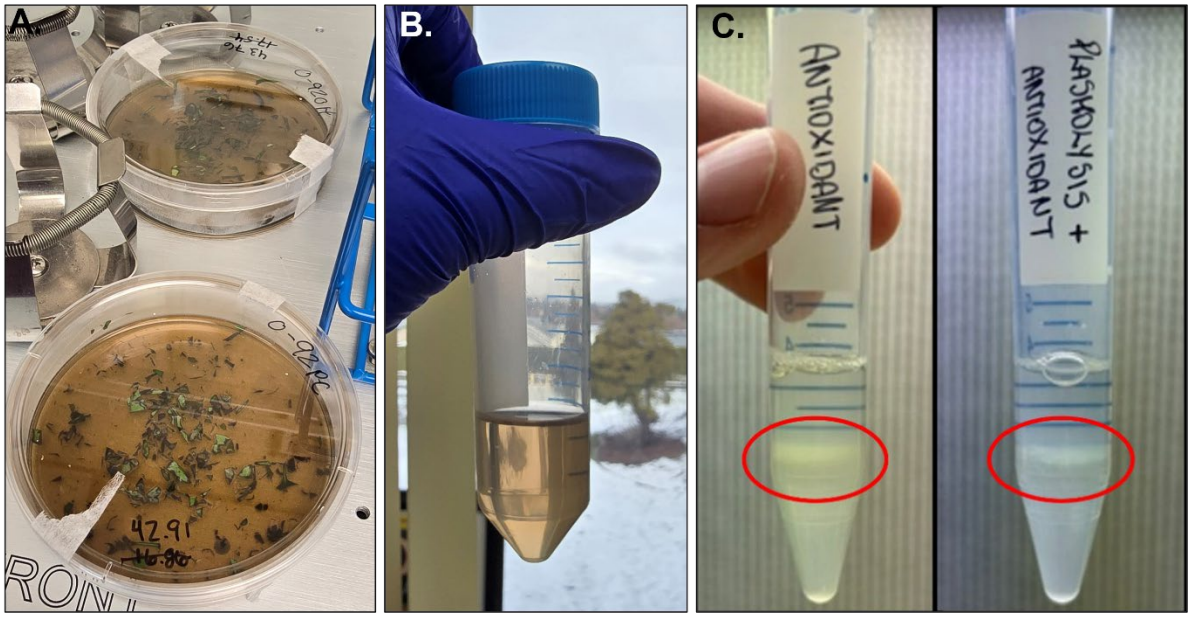

Figure 2. Overdigestion and oxidation during protoplast isolation experiments. A-B. Overdigested and browned tissues were seen after 16-18hours of treatment with all enzyme mixtures tested. Characteristic protoplast layer was no seen in B., compared to treatments from 2023 in C.

Objective 3: Design and generate 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, such that once we develop a reliable protoplast regeneration system we will be 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. 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), and used the program JBrowse to determine that the genes were correct and expressed [8]. 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 [9]. 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)

Discussion

Over the years of this project, funded by both the Fresh and Processed Pear Committees and the California Pear Advisory Board, we have learned a great deal and made significant improvements to callus production and phenotypes, adventitious shoot regeneration (in the absence of Agrobacterium),

and protoplast isolation in ‘Bartlett’, ‘OHxF 87’ and ‘OHxF 97’ germplasm. This foundational knowledge is absolutely crucial for building biotechnological tools like a transgene-free gene-editing system. Tissue culture-based techniques like micropropagation, adventitious shoot regeneration, transformation, protoplast isolation, and rooting were developed decades ago. However, work with these techniques in tree crops like pears has been done by relatively few researchers, and many protocols have not been revisited for a long time. Until quite recently in this field of *in vitro* biology, institutional knowledge was not often published and much has been lost as researchers retire. Further, many older articles were published before high-quality photographs were typically included in journals, which makes it more difficult to reproduce protocols. Thus, building this knowledge anew, and especially with cultivars relevant to the U.S. pear industry, has helped us to take great steps toward developing gene-editing in pears.

References

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Executive Summary

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

Keywords: adventitious shoot regeneration, protoplasts, DNA-free transformation

Abstract:

Gene editing has a strong potential to be useful for clonal crop species like pears. This is in part because it allows for the ability to make precise DNA changes without breeding, which gives us an additional tool for introducing traits into the germplasm. However, traditional gene-editing relies on the integration of transgenes into the plant's genome. Methods for the removal of transgenes often require additional rounds of breeding, especially for clonal species, which counteracts many of the benefits. In the past decade, researchers have begun developing methods for transgene-free gene editing in many crop plants, in which gene-editing machinery is introduced into plant cells without integrating any foreign genetic material into the plant's DNA. This reduces the need for additional rounds of breeding to address regulatory concerns. One of the most common ways this has been achieved is through introducing gene-editing machinery into protoplasts cells to edit the DNA, allowing these edited cells to grow into callus tissue, and then subsequently regenerating adventitious shoots from those cells. Protoplasts are plant cells which have had their cell walls digested, allowing for easier movement of the gene-editing machinery into the cell to reach the nucleus. The most difficult steps in this process are isolation of protoplasts and culture into callus, and adventitious shoot regeneration from that callus. This is in part due to each pear genotype having specific and distinct responses to media additives like nutrients and hormones. This year, we aimed to improve upon adventitious shoot regeneration protocols for ‘Bartlett’, ‘OHxF 87’ and ‘OHxF 97’ genotypes, optimize protoplast isolations by testing more digestion parameters, and finish designing the gene editing machinery. We focused in on one of our highest-producing shoot regeneration experiments from the previous year – plant growth on rooting media and dark treatment prior to leaf excision – and found that when performed on a larger scale, these treatments alone did not improve adventitious shoot regeneration. However, our efficiency rates were generally higher than they have been in the past, signifying that overall, our base protocols have improved. Protoplast isolation trials were run to test enzyme concentrations, combinations, and digestion times from three previously-published protocols for pears, and all led to overdigestion and oxidation. In future trials, we will expand the concentrations and digestion times with these enzymes, as well as vary the antioxidant concentrations in the media. Finally, specific guide-RNA target sequences of the Phytoene Desaturase gene were identified to be used with the gene-editing machinery once a protoplasts isolation and regeneration system are established. Future work will continue to focus on developing and optimizing protocols for these more difficult steps, such that genotypes important for the U.S. pear industry can be edited for important trait-associated genes.