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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WTFRC Collaborative Costs: none

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

Objective 1: Optimize methods for tissue generation needed for protoplast isolation and plant recovery.
Objective 2: Optimize methods for generating pear protoplasts from *in vitro* tissues.
Objective 3: Design and generate gene-editing machinery and introduce into plant cells.

Significant Findings

- Characterized different callus tissue types in 'Bartlett' and 'OHxF 87' resulting from using different hormone inputs after wounding leaves.
- Optimized adventitious shoot regeneration in 'Bartlett' and partially in 'OHxF 87' and 'OHxF 97'.
- Waite Lab learned protoplast isolation methods from Brown Lab and began applying these techniques in both locations.

Methods

This project was co-funded by both the Fresh and Processed Pear Committees and the California Pear Advisory Board. Work took place in both the Waite Lab in Wenatchee and the Brown Lab at UC Davis. Each lab designed experiments to tackle distinct and overlapping parts to the objectives. The methods and results specify the different experiments done in each lab.

Plant Materials:

For micropropagation in the Waite Lab, 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) which 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, 3% w/v sucrose, 4.4 µM 6-benzylaminopurine (BAP), 0.6% agar (A111, PhytoTechnology Labs, Shawnee Mission, KS, USA) 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). 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 µmol/m2s irradiance.
In vitro shoots of Bartlett pear obtained from the Waite Lab 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.

Tissue regeneration (Obj. 1):

All tissue regeneration experiments in the Waite lab used NN69 with 2% sucrose and 0.8% gellan gum (Gelzan™ G3251, Phytotech Labs) as a base media, unless otherwise noted (Nitsch and Nitsch, 1969). For Experiment 1, Phase 1, recently unfurled leaves from 'Bartlett' and 'OHxF 87' were removed from micropropagated plants and soaked for 1 hour in liquid NN69 media containing 2% sucrose, 10µM NAA, and 22.7µM TDZ. 20-30 leaf discs per treatment were removed from the leaves using a 4mm biopsy punch, placed back in the liquid soaking media until all discs were made and placed on solid media containing 1 of 6 treatments (see Table 1). Leaf discs were punched from the petiole-end of the leaves (2 discs per leaf) and contained midrib tissue, both of which contain tissue

that is more competent to regenerate adventitious shoots. Three replicate experiments were performed. Leaf discs were left in the dark on these treatments at 20C for 30 days and callus quality and shoot regeneration was recorded. For Phase 2, leaf discs were transferred to media containing 4.9µM IBA and 9µM TDZ for a subsequent 30 days and shoot regeneration was recorded, and for Phase 3, leaf discs were transferred to media containing only 9µM TDZ as the cytokinin, without auxin. Regeneration rates were recorded after 30 days. For Experiment 2, leaf discs were harvest and soaked in the same way as for Experiment 1. 16 leaf discs were used per treatment. Leaf discs were placed on 1 of 4 treatments (see Table 2) and placed at 20C in the dark. Leaf discs were examined at 3, 5, 7, 10, 15, 21, and 30 days for callus formation and shoot regeneration. Three replicate experiments were performed, and a fourth replicate was kept in the dark for the full 30 days and examined at the end. After 30 days, leaf discs were transferred to plates containing a lower level of TDZ (4.5µM) only, and regeneration was recorded after an addition 30 days (60 days total). Following these experiments, regeneration has been carried out with the following protocol for Bartlett: leaves removed and soaked 1 hour in liquid NN69 media containing 2% sucrose, 10µM NAA, and 22.7µM TDZ, transferred to solid NN69 media with 2% sucrose, 0.8% gellan gum, 4.9µM IBA, and 9µM TDZ and grown in the dark for 30 days, then transferred to solid NN69 media with 2% sucrose, 0.8% Gelzan, and 4.5µM TDZ and grown in the dark for an additional 30 days.

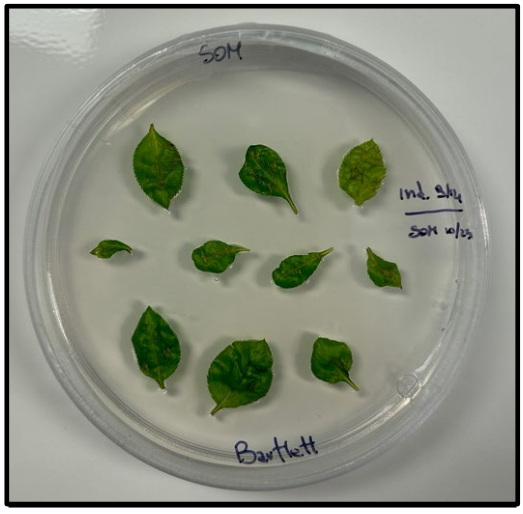
Table 1. Callus Induction Treatments for Exp. 1

		Cytokinin	
		13.6µM TDZ	22.7µM TDZ
Auxin	1µM NAA	T1	T2
	10µM NAA	T3	T4
	4.5µM 2,4-D	T5	T6

Table 2. Regeneration Treatments for Exp. 2

	Auxin	Cytokinin	Base media
Treatment 1	10µM NAA	22.7µM TDZ	NN69
Treatment 2	10µM NAA	22.7µM TDZ	MS
Treatment 3	4.9µM IBA	9µM TDZ	NN69
Treatment 4	4.9µM IBA	9µM TDZ	MS

Shoot organogenesis experiments in the Brown lab were performed by Giuseppe Vaia, a visiting scholar from the University of Tuscia. The first 5-6 apical leaves excised from 3-week-old shoot were used as starting explants for adventitious shoot induction experiments. The adaxial surface of each leaf was randomly wounded with forceps and placed (10 per plate), adaxial side up, on shoot organogenesis medium (SOM) (Figure 1), consisting of MS modified basal medium with Gamborg vitamins (PhytoTech, M404) supplemented with an additional 100 mg/L of myo-inositol, sucrose 3% (w/v), 15 µM of thidiazuron (TDZ) and 1µM 1-napthaleneacetic acid (NAA) (pH 5.7, gelled with 0.6% agar – PhytoTech A111).



Three additional different compounds were tested by adding to the SOM, at the concentration commonly reported in literature: silver nitrate ($AgNO_3$) 10 mg/L, salicylic acid 10 mg/L, and cefotaxime 200 mg/L. Previous works have reported improved plant regeneration using inhibitors of ethylene such as silver

nitrate and salicylic acid (Plus et al., 1993; Chae and Park, 2012; Park et al., 2012). Cefotaxime, is also known to enhance callus growth and plant regeneration. It is assumed that plant enzymes called esterases can break down cefotaxime to new compounds that might have growth-regulating properties. Moreover, it was proposed that cefotaxime might inhibit ethylene production in cultures, which is positively correlated with plantlet differentiation from the callus mass.

Plant protoplast isolation and digestion (Obj. 2):

Protoplast isolations in the Waite Lab were performed using 0.5 g of recently unfurled fully expanded leaves obtained from 'Bartlett' tissue culture plants transferred to and grown for 3 weeks on QL media containing 5µM *meta*-Topolin (*m*T) as cytokinin. Harvested leaves were cut into thin 1-2 cm ribbons and submerged in 5 mL enzymatic digestion buffer. Enzymatic digestion was performed using 1% Cellulase RS, 0.2% Pectinase and 0.2% Macroenzyme R10 dissolved in buffer containing 5mM MES, 10mM CaCl, 11% mannitol as the osmoregulator, 0.1% BSA, and 0.3% glycine (pH 6.0). All enzymatic digestions were carried out at either 22C for 12 or 16 hours or at 25C for 6 hours on a rotary shaker at 40 rpm. The digest solution was filtered through a 45µm nylon mesh filter (Sigma) into 50 mL conical tubes. Protoplasts were separated from the digestion solution by centrifugation at 100xg for 10 mins at 22C. Purification of protoplasts was performed as previously described elsewhere (Ochatt and Power, 1988) using a modified CPW buffer containing 5 mM HEPES, 100mM CaCl, 40uM glycine and 600uM mannitol containing 200 uM BSA. Protoplast were visualized using Evans blue staining on an Olympus BX53 microscope and Olympus DP74 attached camera, using the 40x objective and 10x ocular (for a total of 400x).

Protoplast isolations in the Brown lab were also performed by Giuseppe Vaia. The first attempt followed the grape protoplast isolation protocol described by Tricoli (2019), however, there were incomplete digestions and the tissues browned quite badly. To fight the enzymatic browning, we tested plasmolysis, which involves using a high-solute-containing solution to allow the cell membrane to pull away from the cell wall. Tissue was soaked for 1 h in 3 ml of osmotically adjusted washing solution (WS) containing 0.6 M mannitol, 3 g/L Glycine, 2mM $CaCl_2$, 0.1% bovine serum albumin (BSA) and 0.12% HEPES, and the addition of a modified Tricoli (2019) antioxidant mix (AOx) to the enzyme solution. The antioxidant mix consisting of 0.1% ascorbic acid, 0.15% sodium citrate dihydrate, 0.1% N-Acetyl-L-cystein and 0.03% L-Glutathione reduced.
Protoplasts of 'Bartlett' were isolated from tissue derived from shoot organogenesis pre-conditioning, obtained as described above. These tissues were primarily callus but contained some leaf tissue. Approximately 0.5 g of material was collected and then sliced with a scalpel blade and immediately transferred to a 3 ml of a cell-wall digestion enzymatic solution composed by 0.5% Cellulase Onozuka RS, 0.25% Macerozyme R10, 0.25% Pectinase, 1% BSA, 5 mM $CaCl_2$, 5 mM 2-(N-morpholino) ethane sulfonic acid (MES), 3% Glycine and 0.6 M mannitol, pH adjusted to 6.0 (Tricoli, 2019), the solution was filter-sterilized with 0.2 µm nylon mesh.
The containers (Nalgene screw-top) were placed in a rotary shaker at room temperature in the dark at 50-60 rpm overnight. After approximately 16 hours incubation, the protoplast solution was filtered through a 40 µm screen and the protoplasts were collected by pelleting via centrifugation at 350 rpm (26 *g*) for 10 minutes. The supernatant was discarded and the pellet of protoplasts was slowly re-suspended in 3 ml of osmotically adjusted washing solution (WS), after they were centrifuged again at 350 rpm for 10 minutes.
Protoplasts were purified using a dextran gradient consisting of 2 ml of a 13% dextran solution, containing also 0.4 M sucrose, 2mM $CaCl_2$, 0.1% BSA and 0.12% HEPES, overlaid with 2 ml of 0.6 M WS. Protoplasts in dextran gradient were then centrifuged at 350 rpm for 8 minutes. The ring of viable protoplasts, visible in the layer interface, was aspirated by using a Pasteur pipette.

Gene editing machinery and methods for introduction (Obj. 3):

We have identified several companies that manufacture ribonucleoproteins (RNPs) for delivering CRISPR machinery into plant cells. We will be using a Phytoene Desaturase (*PDS*) gene as an initial target, as resulting plant material is identifiable by its white tissue, due to a lack of chlorophyll. The specific PDS gene in pear we will target is Pycom04g02050.

RESULTS AND DISCUSSION

Tissue Regeneration

In both labs, culture of leaf explants showed 100% callogenesis (growth of callus) after 4 weeks, regardless of regeneration media or leaf explant type (full leaves or discs), concentrated particularly in

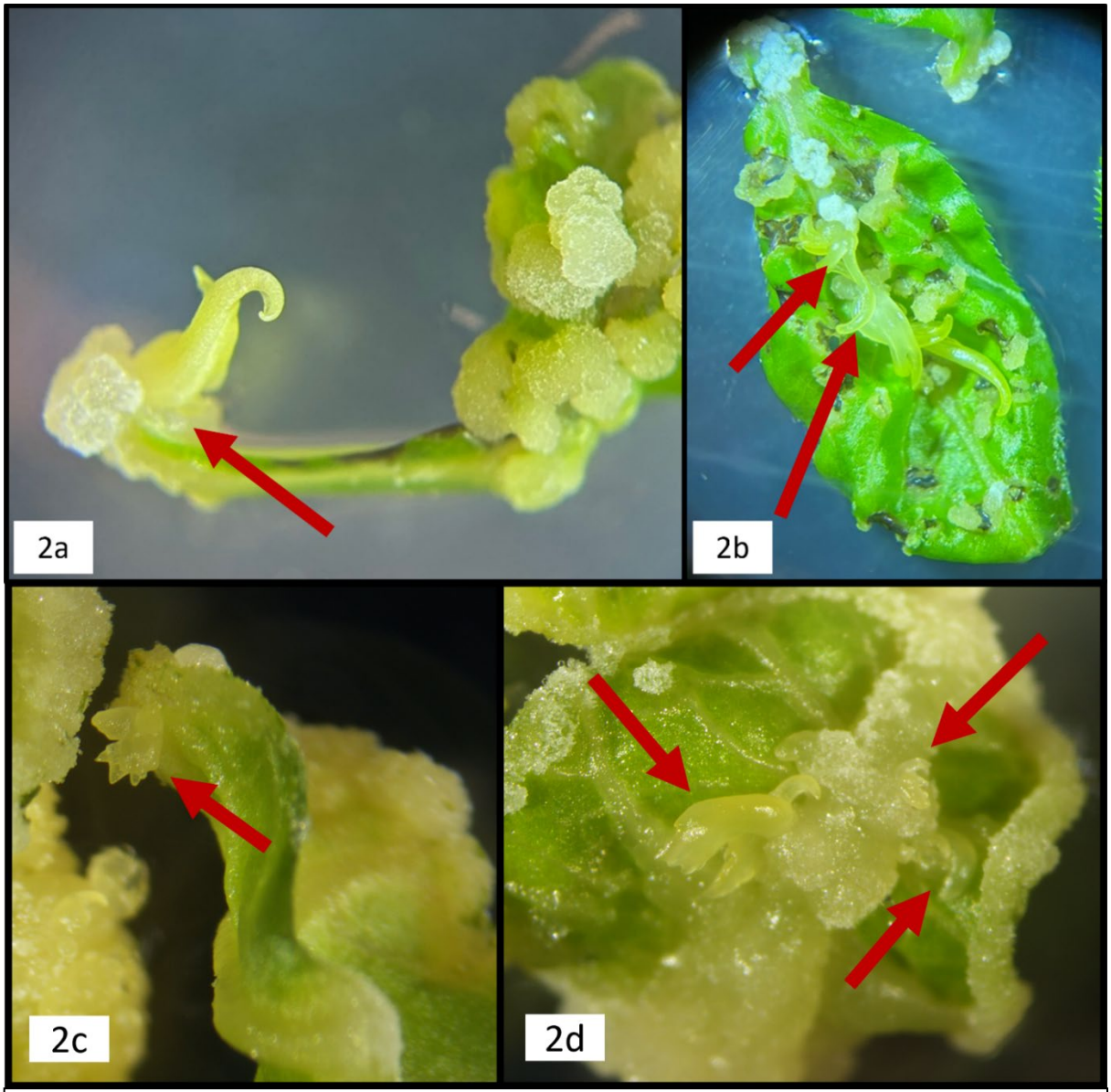


Figure 2. Adventitious shoots forming on full leaf explants (2a and b) and leaf discs (2c and d). Shoots are localized in the petiole (2a) and midrib (2b and d) areas, and occasionally at wound sites on leaf discs (2d).

the petiole, midrib and wounded areas. However, the regenerated shoots developed mainly in the petiole and midrib area (Figure 2), concentrated in the proximal area of the leaf, while adventitious buds were rarely or never observed in the wounded areas.

The Waite lab worked toward optimizing tissue regeneration from leaf discs, starting with 'Bartlett' and 'OHxF 87'. Previously, callus production on 'Bartlett' leaf discs occurred on all leaf discs, but the efficiency of regeneration of adventitious shoots from that callus was low – less than 10%. Note that for experiments in the Waite Lab, regeneration efficiency is reported as total number of adventitious shoots divided by total number of leaf discs, x100, which does not account for average number of shoots per leaf disc, which we will record for all future experiments. To better understand how different hormone types, levels, and combinations affected callus formation and quality, we performed an experiment subjecting 'Bartlett' and 'OHxF 87' leaf discs to six different hormone

		Phase 1		Phase 2		Phase 3	
		Average	SEM	Average	SEM	Average	SEM
Bartlett	T1	4.68	0.37	2.92	1.48	2.92	1.48
	T2	1.39	1.39	11.46	4.89	1.75	1.75
	T3	3.03	3.03	12.24	3.06	4.78	2.64
	T4	4.35	4.35	12.97	2.32	2.78	1.39
	T5	0	0	1.67	1.67	0	0
	T6	0	0	0	0	0	0
OHxF 87	T1	1.33	1.33	0	0	5.22	0.78
	T2	4.33	2.33	1.33	1.33	9.11	2.73
	T3	4.70	2.63	1.52	1.52	7.73	5.42
	T4	0	0	0	0	11.21	5.90
	T5	0	0	0	0	8.02	3.14
	T6	0	0	0	0	2.98	1.50

Table 3. Regeneration Rates for Phases 1, 2, and 3 of Experiment 1. Regeneration rates were calculated as total number of shoot regenerants divided by total number of leaf discs, x100 for percent values. Average of three replicate are presented, with standard error of the mean reported. Yellow highlights represent the three highest rates for each cultivar.

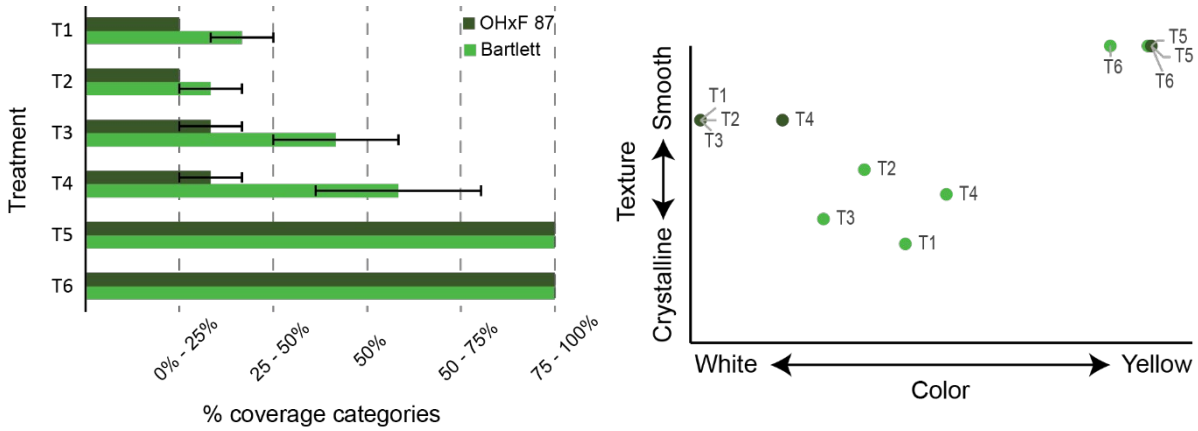


Figure 3. Callus growth/coverage and quality in response to hormone treatments. (Left) Average percent of leaf discs that were covered by callus tissue 30d after growth on the six different hormone treatments (Table 2). (Right) Texture and color of callus grown on different hormone treatments were observed, assigned a number category, and averaged across replicates. Dark green dots represent OHxF 87 on the six different treatments, light green dots represent Bartlett.

treatments (Table 1). Callus coverage and quality parameters were measured, as well as any shoot regeneration during the first 30 days (Figure 3 and Table 3). From this part of the experiment, we noted that treatments 1, 3, and 4 were highest for 'Bartlett' and had similar regeneration rates (4.7, 3.0, and 4.3%, respectively), and treatments 2 and 3 were highest for 'OHxF 87' (4.3 and 4.7%, respectively).

We performed a literature review covering regeneration from pear callus tissue, and identified a hormone treatment that had performed well for another group developing protocols for Pyrodwarf (4.9µM IBA and 9µM TDZ) (Vujovic et al., 2014). To test whether the callus types generated from Experiment 1, Phase 1 could regenerate equally in response this treatment, leaf discs were transferred to media containing these hormones and grown for an additional 30 days (Table 3). 'Bartlett' callus generated on Treatments 2, 3 and 4 responded well to this treatment, whereas any callus generated on the auxin 2,4-D (Treatments 5 and 6) showed almost no regeneration. 'OHxF 87' callus generated on all treatments showed low to no regeneration during Phase 2.

Our literature review also revealed that some groups have had regeneration success transferring callus tissue onto plates containing only cytokinin and no auxin (Leblay et al., 1991; Caboni et al., 2002; Bell et al., 2011). To test whether this would have a positive effect on our callus tissue, we performed one final transfer onto 1/2x MS media containing 9µM TDZ only. These plates contained either sucrose or sorbitol as a carbon source, but we saw little difference between these, and thus combined results are reported (Table 3). We saw some additional regeneration in 'Bartlett', for callus that was originally generated on Treatments 1-4. 'OHxF 87' rates increased, particularly for callus generated on Treatments 2-5. It is possible that this is a response to the treatment in Phase 3, or signifies delayed regeneration, as compared to 'Bartlett'. This question will require further exploration.

Based on these results, we decided to compare the best performing treatments from Experiment 1 (Treatment 4 from Phase 1 (10µM NAA and 22.7µM TDZ) and the hormone combination from Phase 2 (4.9µM IBA and 9µM TDZ)) with different base medias (NN69 and MS), and look at callus formation and shoot regeneration over time in each cultivar. Leaf discs were grown on these treatments (Table 2) for 30 days, observing callus formation at 3, 5, 7, 10, 15, and 30 days, and then

				Regen. @ 30d on treatment		Additional regen. after 30d on TDZ-only	Total regen. rates after 60 days
	Auxin	Cytokinin	Media	Avg	SEM	Avg	Avg
Bartlett	10µM NAA	22.7µM TDZ	NN69	0	0	18.75	18.75
			MS	0	0	6.25	6.25
	4.9µM IBA	9µM TDZ	NN69	15.63	4.03	71.88	87.50
			MS	7.81	2.99	42.19	50.00
OHxF 87	10µM NAA	22.7µM TDZ	NN69	0	0	0	0
			MS	0	0	0	0
	4.9µM IBA	9µM TDZ	NN69	9.38	1.80	26.52	35.90
			MS	7.81	1.56	23.89	31.71

Table 4. Regeneration rates for Experiment 2. Regeneration rates were calculated as total number of shoot regenerants divided by total number of leaf discs, x100 for percent values. Average of three replicate are presented, with standard error of the mean reported. At the 60 days timepoint, data from all 4 replicates (16 leaf discs each) was pooled, so standard error could not be calculated. TDZ-only plates contained 4.5uM TDZ and no auxin.

transferred to TDZ-only plates, this time with a lower concentration (4.5µM TDZ), and grown for an additional 30 days. For both 'Bartlett' and 'OHxF 87', growth on NN69 media containing 4.9µM IBA and 9µM TDZ, followed by transfer to 4.5µM TDZ, resulted in the best regeneration rates (Table 4). These same hormone combination with MS base media also performed well, but to a lesser extent. Since these experiments, we have continued use of the two-phase protocol, starting with 30 days on NN69 with 4.9µM IBA and 9µM TDZ, followed by transfer to 4.5µM TDZ for 30 more days, and have regularly seen 80-90% regeneration rates for 'Bartlett'. Again, this rate calculation represents total number of shoots per total number of leaf discs, not accounting for number of shoots per leaf disc, which we will record in future experiments. We noted that for this second TDZ-only phase, some leaf discs had multiple shoot per disc, while others had none.
These findings have helped us in regenerating and producing the tissue we need to isolate protoplasts, and knowledge of an optimized hormone combination for regeneration in 'Bartlett' will further be useful in regenerating tissue from the protoplasts themselves.

Results from the Brown Lab's experiments comparing regeneration capacity of the explants excised from rooting media and those excised from the multiplication media were significant, while the three media modifications (addition of silver nitrate, salicylic acid, or cefotaxime) showed no improvement over the standard organogenesis medium. Regeneration efficiency (calculated by the number of leaves with at least one shoot per total explants x 100) was more than 35% for leaves from the rooting media, while for leaves from the multiplication media it did not exceed 3%. Nevertheless, no difference has been observed about the average number of shoots per regenerating leaf that was around one/two, with some exceptions even up to three. Previous papers showed that regeneration capacity is strictly linked to pear genotype, and our results seem to be in line with those reported in the same cultivar (Yousefiara et al., 2014). In addition, the data from cited articles was measure 8 weeks after wounding (as well as other related articles), so we expect a continued increase in the number of shoots forming in the coming weeks.
Further studies in the Brown lab will focus on different hormones concentration and type of salts in the regeneration medium, since has been reported that ammonium/nitrate ratio were essential in shoot regeneration of pear (Leblay et al., 1991).

Plant protoplast isolation and digestion

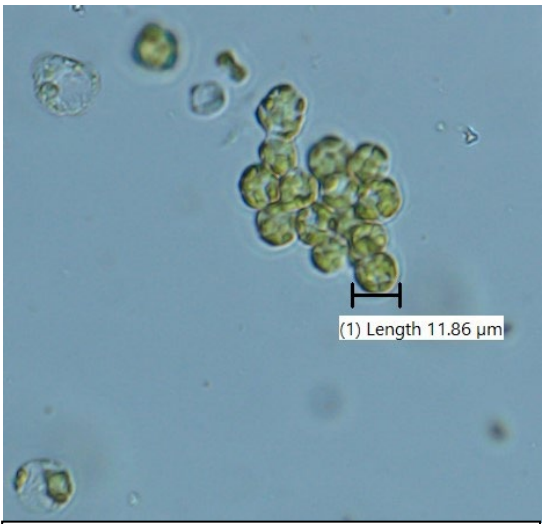


Fig 4. Bartlett protoplasts isolated in Waite Lab.

Members of the Waite lab were able to use CPAB funding this year to travel to the Brown lab at UC Davis and learn protoplast isolation protocols, resulting in both labs now being able to work towards this goal. Pear tissues have been difficult to fully digest and isolate protoplasts from. As a result, trials varying the digestion buffers and duration of digestion were performed. Digestions in the Waite lab carried out at 22C for 16 hours and 12 hours resulted in no visible protoplasts or non-viable protoplasts, respectively. Digestion at 25C for 6 hours yielded greater number viable protoplasts that were incompletely digested (Figure 4).
Results from the Brown Lab showed that addition of antioxidants improved protoplast isolations, resulting in a mixture that was clear and almost free of impurities and debris (Figure 5, right tube compared to left tube). This might be due to the production of phenolic compounds, which might substantially affect

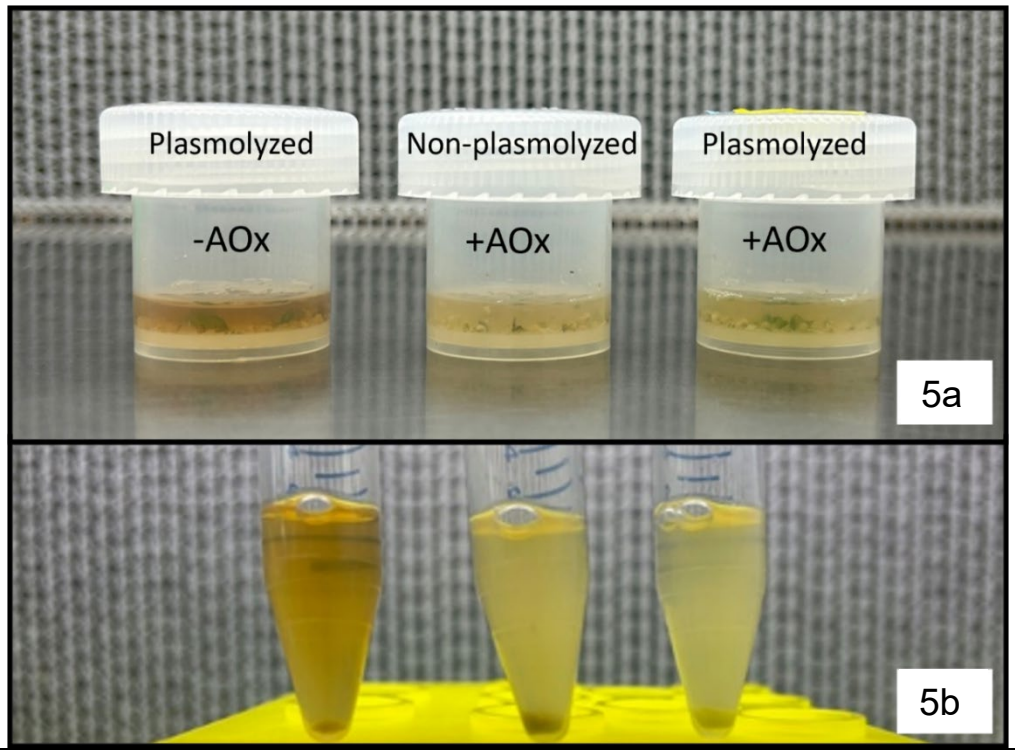


Figure 5. Cell-wall digestion enzymatic solution after 16h incubations (5a) and after the first centrifuge (5b). From left to right are the plasmolysed sample without antioxidant mix in the enzymatic solution, the non-plasmolysed sample with antioxidant mix in the enzymatic solution and the plasmolysed sample with antioxidant mix in the enzymatic solution.

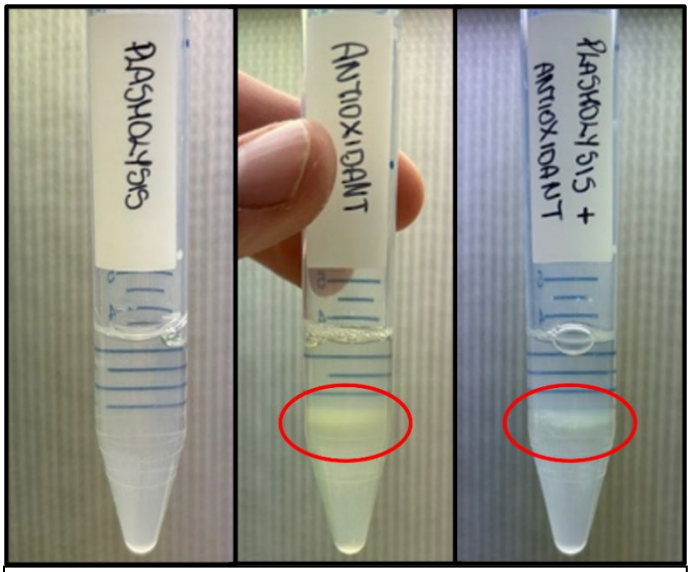


Figure 6. The ring of protoplasts, visible in the layer interface, after the centrifugation in dextran gradient.

the digestions of the cell walls. Indeed, after centrifugation, there were no visible protoplasts in the solution obtained without the antioxidant mix (Figure 6). When plasmolysis was tested (using high-solute solutions to separate the cell membrane from the cell wall), no differences were observed between plasmolysed and non-plasmolysed samples in terms of the solution color after 16 h incubations (Figure 5a) or the amount of protoplast visible in the layer interface after the dextran gradient (Figure 6). The protoplasts were harvested and counted using a counting chamber. The yield of the harvested protoplasts was around 1 x 10^6 cells per ml in the samples with the antioxidant mix in the enzymatic solution (Figure 6). These results showed the crucial importance of adding an antioxidant mix to the enzyme solution to prevent browning due to phenol production and achieve an improved protoplast yield.

Gene editing machinery

This year, we researched the specific *PDS* gene we will use as a gene-editing target. Starting with *PDS* as a target allows for quicker assessment of whether the gene-editing system is functional, as knocking out this gene results in white tissue as soon as plants are regenerated. The *PDS* gene in the Bartlett genome we will target is Pycom04g02050, which has been recently targeted in pear gene-editing, using a DNA-integrated system (Malabarba et al., 2021). Further, we have researched and found several biotechnology companies that manufacture CRISPR RNPs for gene editing that we can use for delivering the gene editing machinery, once we have generated protoplasts in the quantities needed for transformation.

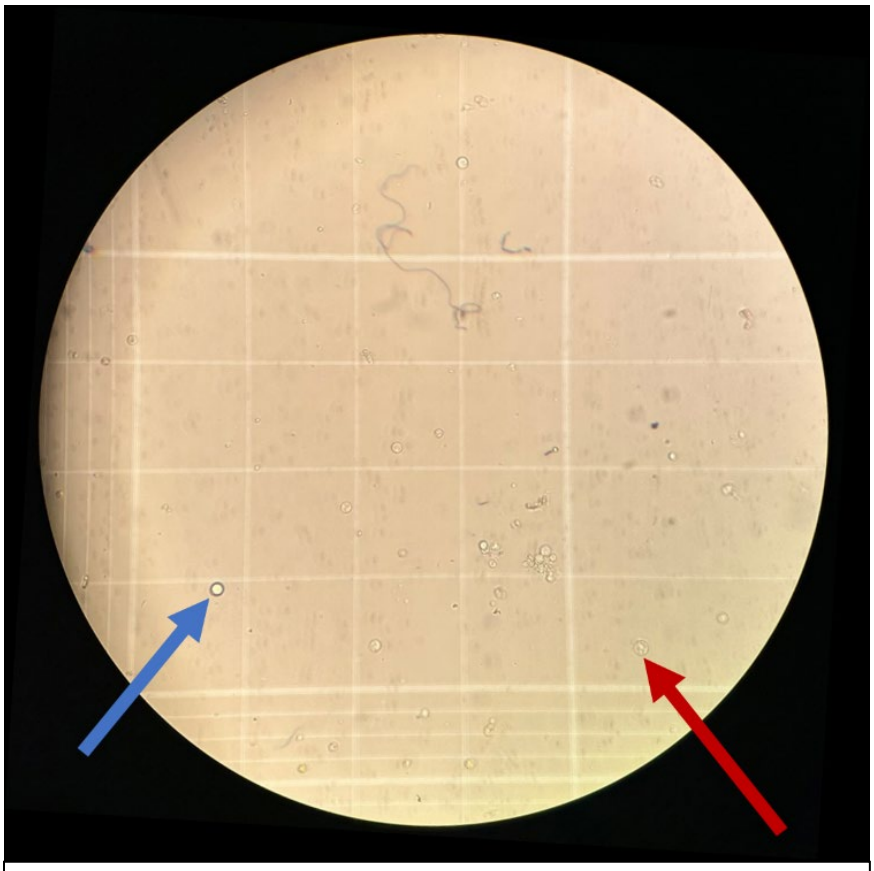


Figure 7. Counting of viable protoplasts (red arrow) with the help of counting chamber, observed with 100X magnification. 20 µL of protoplast solution have been diluted in 180 µL of washing solution before being counted. The spherical object indicated by the blue arrow is an air bubble and is not counted.

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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. This year, we proposed to lay the groundwork for developing a transgene-free gene editing system in pears. To do this, we focused on optimizing adventitious shoot regeneration from pear callus tissue, began optimization of protoplast isolation from pear tissues, and researched gene targets and synthesis of gene editing machinery. Adventitious shoot regeneration from pear callus tissue was important for two reasons: allowing us to define a protocol for generating callus tissue that is competent to regenerate, and understanding the ideal hormone combinations each cultivar responds to for efficient regeneration. These will help us both to generate tissue for protoplast isolation and to regenerate plants from protoplasts. This year, we were able to screen different hormone combinations and identify an efficient protocol for 'Bartlett' callus formation and adventitious shoot regeneration. While we were able to increase efficiency slightly for OHxF 87 and 97, our future work will focus on improving efficiency for these cultivars. The two collaborating labs were able to meet this year and share methods, such that both groups have now begun the work of optimizing protoplast isolation. Our attempts thus far have narrowed the cell-wall digestion lengths but have struggled with partial digestion or oxidation issues. Future work will focus on testing different cell-wall digestion enzymes and concentrations, solution characteristics, and tissue sources. Finally, we identified the specific pear *PDS* gene and genetic sites to be targeted, as well as researched companies that can synthesize the RNPs we will use to introduce the gene-editing machinery into plant cells. All together, we took significant steps towards developing a transgene-free gene-editing system for pears and will continue working towards building this tool.