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

PIs: Jessica Waite, USDA-ARS, Wenatchee, WA; Pat Brown and Charles Leslie, UC Davis, Davis, CA

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). In the past year, we have made significant progress towards regenerating the tissue needed for these types of tools, as well as early success in generating protoplast from these tissues. This year we propose to optimize these protocols and introduce gene-editing machinery into plant cells.

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 methods for tissue regeneration needed for protoplast generation.

Objective 2: Optimize methods for generating pear protoplasts from in vitro micro shoots.

Objective 3: Design and generate gene-editing machinery and introduce to plant cells.

PROCEDURE

<u>Plant Materials:</u>

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.

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.

Tissue regeneration (Obj. 1):

Shoot organogenesis experiments in the Brown lab were performed by Giuseppe Vaia, a visiting scholar from the University of Tuscia. The first five-six apical leaves excised from 3-week-old shoot were used as starting explants for *in vitro* 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).

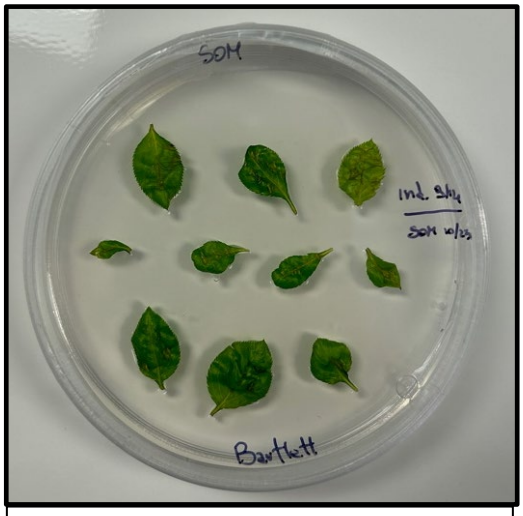

Figure 1. Leaf explants on the shoot organogenesis media (SOM).

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.

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 leaf discs 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, place back in the liquid soaking media until all discs were made and placed on solid media containing 1 of 6 treatments (see Table 1). 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

Plant protoplast isolation and digestion (Obj. 2):

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, 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.

Protoplast isolations in the Waite Lab were performed using 0.5 g of recently unfurled fully expanded leaves obtained from 3 week Bartlett old tissue culture plants grown 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).

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

Our original plan was to build vectors that would contain the gene-editing/CRISPR machinery, and use these to target the chlorophyll biosynthesis gene phytoene desaturase (PDS) for proof of concept. While we still plan to begin with this target, more recent research led us to an alternative method for delivery, which more and more crop systems are using: Ribonucleoprotein-mediated delivery (RNPs). RNPs allow introduction of the pre-assembled CRISPR machinery into plant cells, not needing to depend on expression at all, which was initially tested in Arabidopsis, tobacco, lettuce, and rice, and have since been used in apple, grape, and potato (Woo et al., 2015; Malnoy et al., 2016; Gonzalez et al., 2020). Multiple companies feature RNP design services, which we will compare for purchase.

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 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.

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

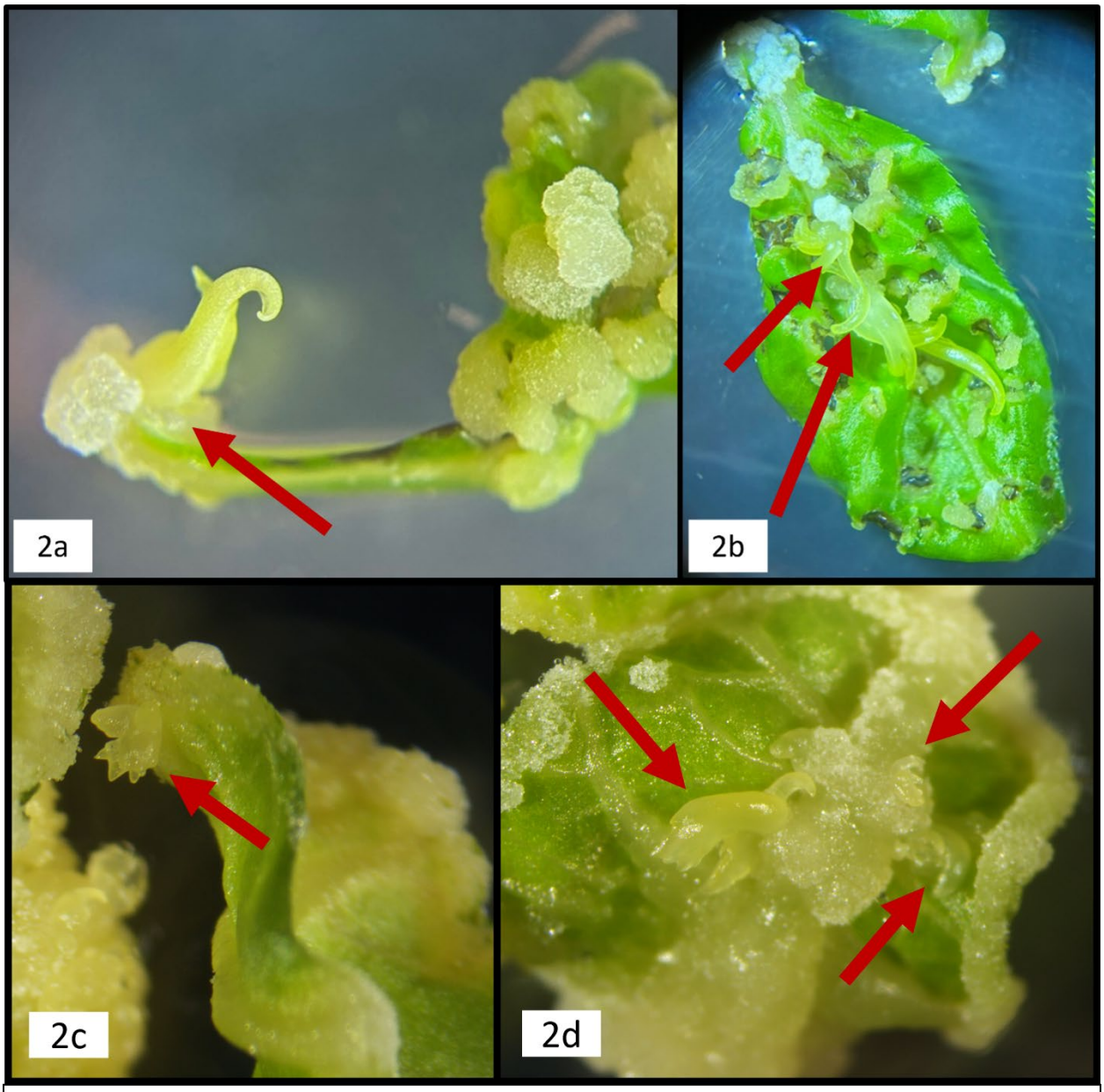

Figure 2. Adventitious shoots forming on full leaf explants (2a and b) and leaf discs (2c and d). Shoots are localized in the petiole (2a) and midrib (2b and d) areas, and occasionally at wound sites on leaf discs (2d).

data from cited article referred to 8 weeks from the leaves culture (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).

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

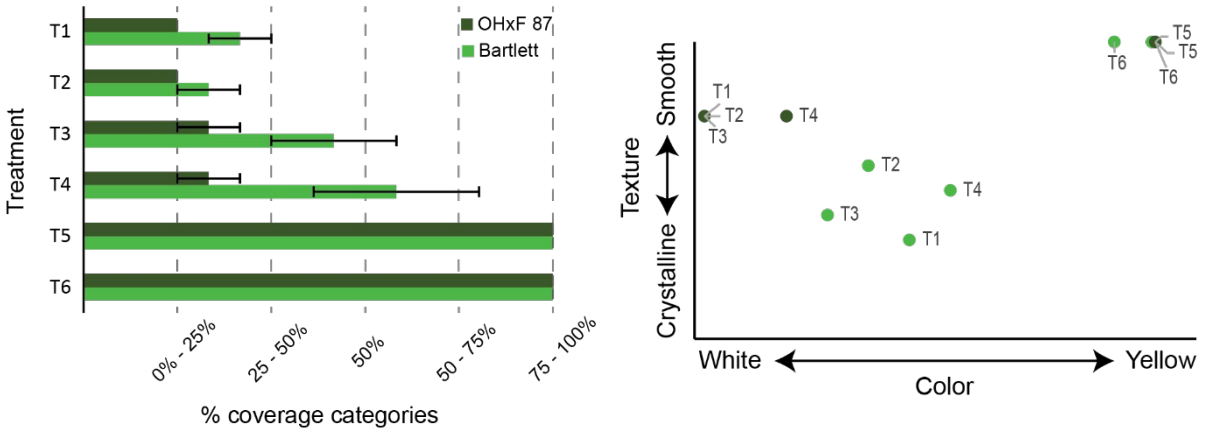

Figure 3. Callus growth/coverage and quality in response to hormone treatments. (Left) Average percent of leaf discs that were covered by callus tissue 30d after growth on the six different hormone treatments (Table 2). (Right) Texture and color of callus grown on the different hormone treatments were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

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 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).

		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.

We performed a literature review covering regeneration from pear callus tissue, identified a hormones 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.

				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.

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 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.

Plant protoplast isolation and digestion

The isolation of protoplasts in the cell-wall enzyme digestion after overnight incubation at room temperature demonstrated that the isolation without antioxidant solution was far more challenging, since the solution appears more brown, in contrast to the solution with antioxidant mix that was clear and almost free of impurities and debris (Figure 4). This might be due to the production of phenolic compounds, which might substantially affect the cell-wall digestion by enzymes and so the protoplast isolation. Indeed, after the centrifugation in dextran gradient there was no visible protoplast in the layer interface of the solution obtained without the antioxidant mix (Figure 5). No differences were observed

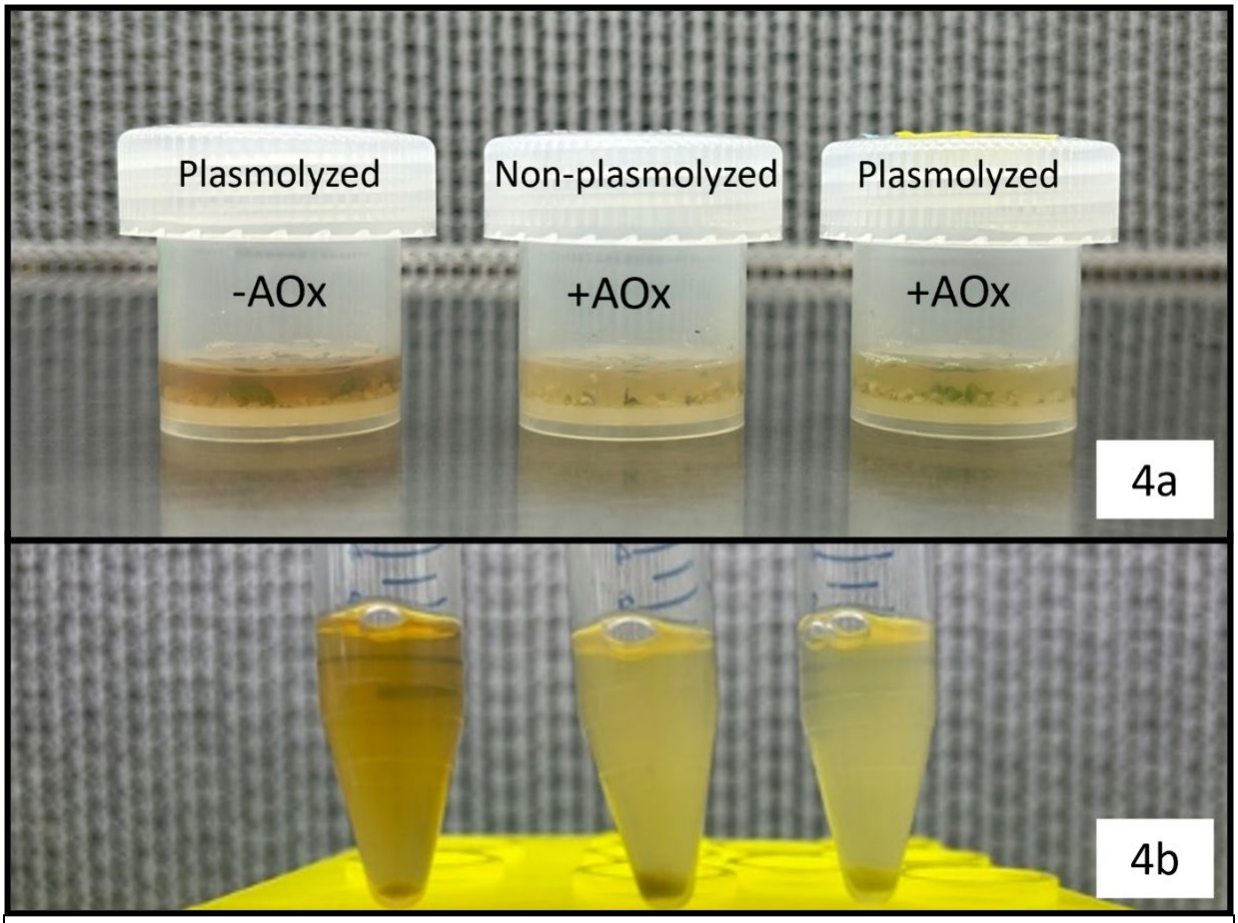

Figure 4. Cell-wall digestion enzymatic solution after 16 h incubations (4a) and after the first centrifuge (4b). From left to right are respectively shown the plasmolysed sample without antioxidant mix in the enzymatic solution, the non-plasmolysed sample with antioxidant mix in the enzymatic solution and the plasmolysed sample with also antioxidant mix in the enzymatic solution.

between plasmolysed and non-plasmolysed samples in terms of the solution color after 16 h incubations (Figure 4a) and the amount of protoplast visible in the layer interface after the dextran gradient (Figure 5).

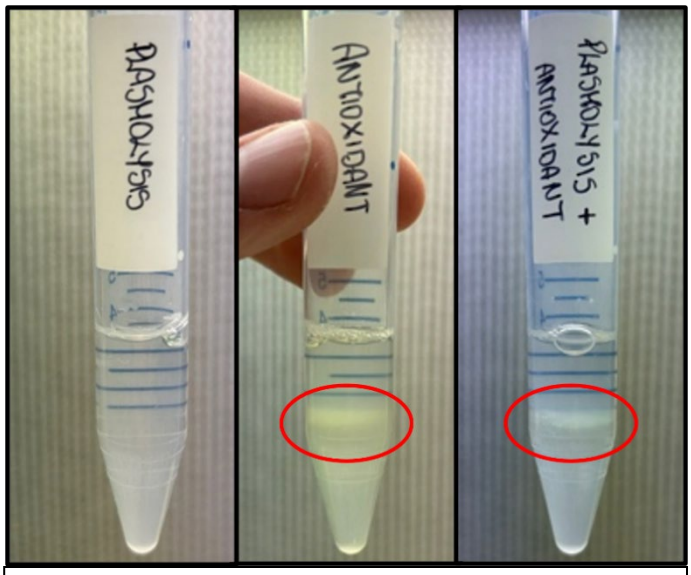

Figure 5. The ring of protoplasts, visible in the layer interface, after the centrifugation in dextran gradient.

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

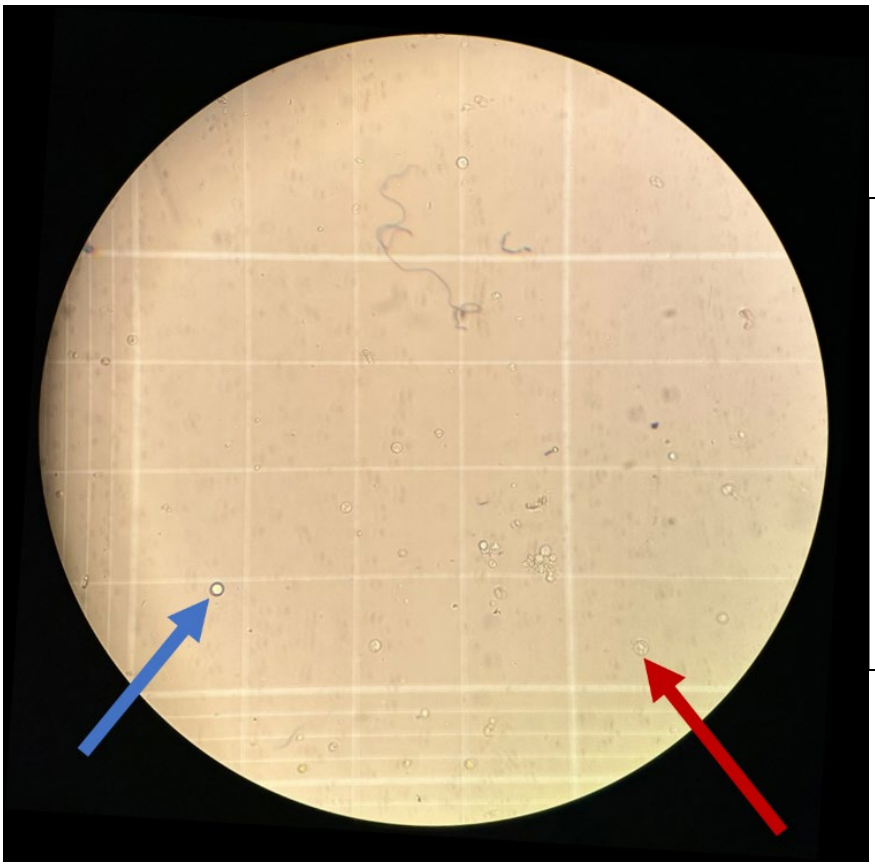

Figure 6. Counting of viable protoplasts (red arrow) with the help of counting chamber, observed with 100X magnification. 20 µL of protoplast solution have been diluted in 180 µL of washing solution before being counted. The spherical object indicated by the blue arrow is an air bubble, and is not counted.

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 a good protoplast yield.

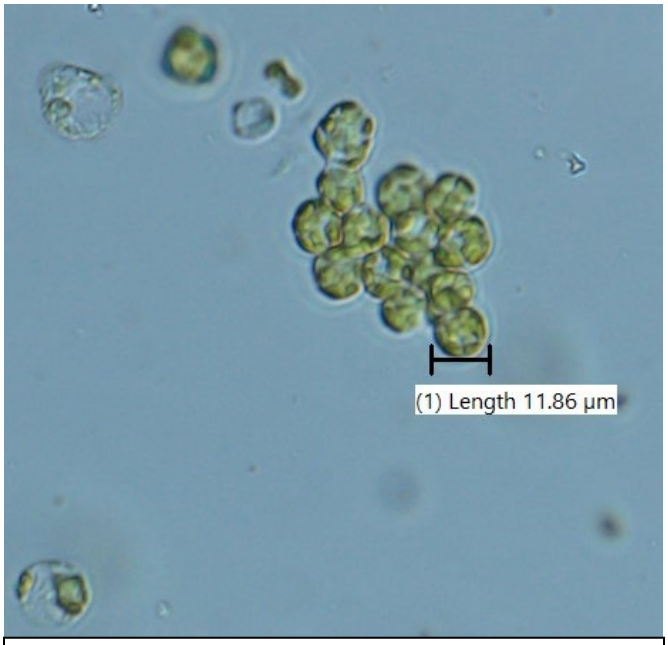

Figure 7. Bartlett protoplasts isolated in Waite Lab.

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 7).

Gene editing machinery

This year, review of new literature changed our plans for developing gene editing machinery. Initially, we planned to develop DNA vectors that, once introduce into the protoplasts, would use the plant's cellular systems to transcribe and fold the gene editing machinery. This is a method that has been used in the past by many groups (Goralogia et al., 2021). However, it is becoming more and more common in woody plants to use Ribonucleoproteins (RNPs) for gene editing with protoplasts (Malnoy et al., 2016; Goralogia et al., 2021; Najafi et al., 2023). RNPs are pre-assembled and folded gene editing machinery, which allows researchers to remove the step of depending on the plant cells to assemble and fold. Additionally, RNPs are developed by many biotechnology companies now, and can be easily designed and purchased. When we have optimized protoplast isolation, multiplication, and regeneration, then we will purchase RNPs to be introduced into protoplasts via PEG-mediated transformation.

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