Preliminary Protocol for High throughput Protoplast Transformation

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Plant preparation:

- Plant ~100 W23 seeds in greenhouse supplied soil
- Water M,W,F with incubator set at 16 hr day light cycle
- On the 5th day, transfer to dark incubator
- Harvest on the 10th day

Protoplasts Preparation:

Preparation of Solutions

- Protoplasting Solution: Set out enzymes (Cellulase/Pectinase) at room temperature for ~30 min. Weigh out enzymes and put into 50 mL conical(s). Return enzymes to 4°C. Pour the protoplasting solution (X- enzymes) into the 50 mL concial. Spin down for 5 min at 2000g in swinging bucket centrifuge. Can weigh out several preps of these enzymes and store aliquots in 4 degree, but protoplast solution should be made the day of.
- Incubation/MMG solution: Can make these the day(s) before the experiment.
- PEG 4000: Make this solution the day of experiment. Weigh out PEG 4000, add to 50 mL conical, add specified amounts (refer to below table) mannitol and CaCl2. Vortex heavily. Top to 15 mL with water. Allow to shake for several hours. Once solution is clear, fill to 20 mL with water. PEG should be prepared the day of the experiment.

Protoplast Soluiion

	A	B	C	D	E	F	G	H
1	**Ingredient**	**Stock Concentration**	**Working Concentration**	**Amount of Each Component (ml)**				
2	**MES - PH 5.7**	0.1	0.01	5				
3	**Mannitol**	1	0.4	20			**Total Volume**	50
4	**Cellulase**	X	0.1% w/v	X	** Add Cellulase and Pectinase to protoplasting solution right before putting the sliced leaves in it.	** For 20 ml - it is 0.02 g		
5	**Pectinase**	X	0.01% w/v	X	***	** For 20 ml -- it is 0.002 g		
6	**CaCl2**	1	0.001	0.05				

PEG 40%

	A	B	C	D	E	F	G
1	**Ingredient**	**Stock Concentration**	**Working Concentration**	**Amount of Each Component (ml)**			
2	Mannitol	1	0.2	6		**Total Volume**	30
3	CaCl2	1	0.1	3			
4	PEG 4000	40% w/v		X	For 30 ml of 40% PEG - it is 12 g		

MMG Solution

	A	B	C	D	E	F	G
1	**Ingredient**	**Stock Concentration**	**Working Concentration**	**Amount of Each Component (ml)**			
2	MES (PH 5.7)	0.1	0.004	20		**Total Volume**	500
3	Mannitol	1	0.4	200			
4	MgCl2	0.1	0.015	75			

Incubation Solution with MgCl2

	A	B	C	D	E	F	G	H
1	**Ingredient**	**Stock Concentration (M)**	**Working Concentration (M)**		**Amount of Each Component (ml)**			
2	MES (PH 5.7)	0.1	0.004		20		**Total Volume**	500
3	Mannitol	1	0.4		200			
4	KCl	1	0.004		2			
5	MgCl2	0.1	0.002		10			

Protoplasting:

1. Place the leaf cuttings in a petri dish containing 20 mL of protoplasting enzyme solution. I would recommend not doing more than ~25 leaves per petri dish (150 X 15 mm petri dish used)

-- If i need ~15 ml of protoplast solution at normalized concentration, I would use ~100 seedlings

1. Can go ahead and place leaf cuttings in the protoplast solution while slicing the other leaves. Lightly brush the slices into each petri dish. Make sure that the slices are covered with protoplasting solution, this can be done by pipetting some protoplasting solution from the dish and carefully repipetting it on slices. Also, a pipette tip can be used to spread out clumps of slices to ensure even protoplasting.
2. Before putting the leaf cuttings in the shaker, vacuum filtrate the cells for 30 minutes in the dark.

[CRITICAL] Make sure that the plate(s) are sitting on a flat surface within the vacuum manifold (a freezer storage box can be used) and that once the vacuum step is completed, the tubing isn't immeaditley moved. Rather, slowly remove the tubing connection to allow for pressure to be released.

1. Incubate at 28°C in the dark with gentle shaking (40 rpm) for two hours
2. Incubate at 28°C in the dark with shaking at 85 rpm for 10 min.
3. Place 50 ml conical(s) on ice. Recommend one conical/petri dish
4. Put 40 um mesh into the 50 ml conical. Pour 2 to 3 ml of MMG into the strainer to wet it.
5. Use a serological pipette to remove the cell solution from the Petri dish, this should be done with care to not exert excess force on the protoplasts (i.e. slowly uptake cells using the serological pipette and slowly dispense through the strainer).
6. Strain the released cells through a 40 um mesh into a 50 ml conical
7. Pour ~5 ml MMG into the plate that had the protoplasts and swirl. Use a serological pipette to remove the cell solution from the Petri dish, this should be done with care to not exert excess force on the protoplasts (i.e. slowly uptake cells using the serological pipette and slowly dispense through the strainer).
8. Fill conical containing cells to 40 ml with MMG.
9. Centrifuge at 100 g for 7min in the swinging bucket centrifuge in the lab which is at room temperature.

[PAUSE] It is okay to spin the cells down for a couple more minutes if the solution is still cloudy. There should be a large green pellet at the bottom of the 50 mL conical with a relatively clear supernatant.

MMG Solution/Counting of Cells:

[CRITICAL] Keep protoplasts on ice as much as you can (during washes and when not doing the spins)

1. Remove the supernatant carefully as not to disturb the cell pellet. '

[CRITICAL] I typically remove the supernatant to about the 5 ml mark on the conical to ensure that I get good washes without disturbing protoplasts.

1. Resuspend the cells in the remaining solution by gently swirling, the pellet should come into the solution with ease.
2. Fill the conical containing the protoplasts to 40 ml with mmg.
3. Centrifuge at 100 g for 6 min in the swinging bucket centrifuge.
4. Remove the supernatant carefully as not to disturb the cell pellet.
5. Resuspend the cells in the remaining solution by gently swirling, the pellet should come into the solution with ease.
6. Fill the conical containing the protoplasts to 40 ml with mmg.
7. Centrifuge at 100 g for 5 min in the swinging bucket centrifuge.
8. Remove supernatant so solution is at 5 ml on the conical using the serological pipette. Then using a p1000, remove another 2 ml so the volume tube is at 3 ml.
9. Resuspend the cells in the remaining solution by gently swirling, the pellet should come into the solution with ease.
10. [CRITICAL] You can use a serological pipette to measure this if you need - just make sure to not exert too much force on the protoplasts.. Make note of the total solution in the tube.
11. Count cells in hemocytometer, add extra MMg solution to bring the final cell concentration to 8 X10^5 cells/ mL
12. Count the cells in the sets of 16 blocks, there shoud be four of these. Count and average to get the average cell concentration. Adjust with MMG solution to get the appropiate concentration.

Cell Dispense and Transformation using Multiflo:

1. Add 1.5 ug of plasmid DNA to each well
2. Add 10 ul of normalized (8 X10^5 cells/ mL) to each well (with the multiflo make sure to use the 10 ul wide bore cassette, medium speed)
3. Sit at room temperature for 5 minute
4. Added 30 ul of 27% PEG to each of well (with the multiflo make sure to use the 10 ul wide bore cassette, medium speed)
5. Cover with foil seal
6. Flip plate back and forth 2X to ensure even mix
7. Spin plate at a low speed for 15 seconds
8. Sit at room temperature for 15 minutes
9. Spin plate at a low speed for 45 seconds

PEG made above is 40% - dilute to 27% with 600 mM Mannitol MMG prior to usage - made 30 ml so diluted to 44.4 ml with 600 mM Mannitol MMG

Incubation Solution:

***** Using the Multiflo for all steps in this section *****

1. Aspirate Z118 using washer
2. Add 80 ul incubation solution
3. Delay for 5 minutes
4. Aspirate Z118 using washer
5. Add 80 ul incubation solution
6. Delay for 5 minutes
7. Aspirate Z118 using washer
8. Add 80 ul incubation solution

9. Delay for 5 minutes
10. Aspirate to Z180``using washer
11. Cover with porous cell cover
12. Put in the 28 degree dark incubator for overnight incubation