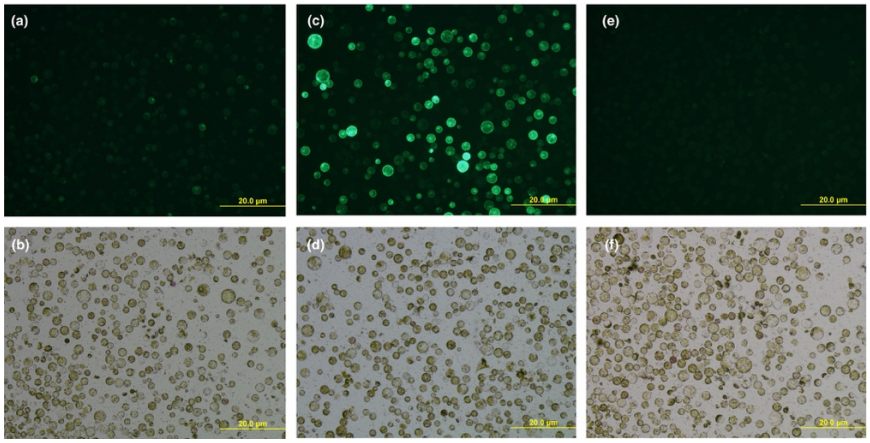
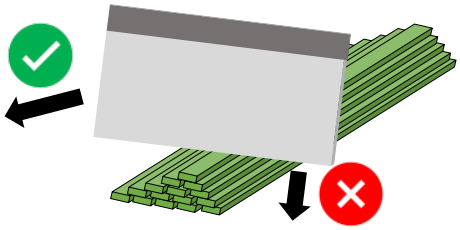
Updated on 07/15/2021

Protoplast Isolation and Transformation in *Tragopogon*



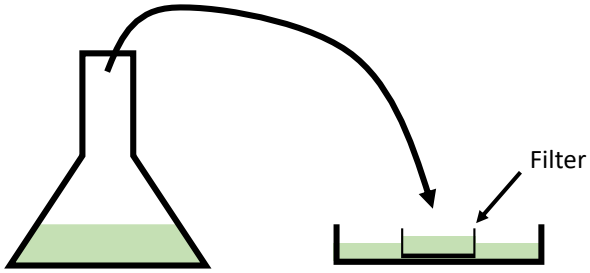
Protocol

1. Prepare D-mannitol Solution
    - Make sure it is fully dissolved; transfer into a sterilized 50 ml flask
2. Prepare the Enzyme Solution
3. Prepare plant material
    - Cut healthy and young *Tragopogon* seedling (2-3 weeks in ½ MS germination medium) leaves into 0.5 mm small pieces
    - Before cutting, blot the leaves on Kimwipes
    - Slice the leaves on a piece of glossy paper (from flyers/magazines) with a blade; do not press the blade vertically

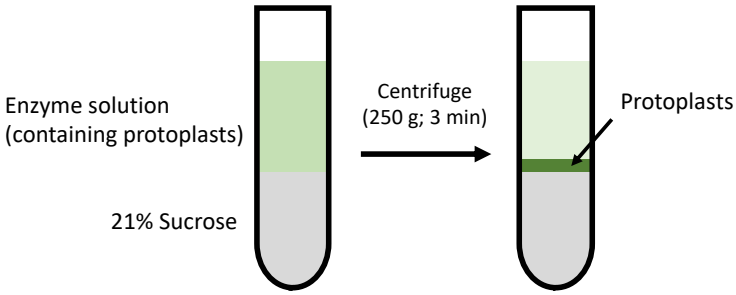


    - To avoid over-dry, transfer the small leaf pieces into the D-mannitol buffer occasionally
4. Transfer the small leaf pieces into D-mannitol solution, and shake at 30-50 rpm, room temperature for ~15 min; the flask is covered by foil
    - Make sure all leaf pieces are in the solution
5. Remove the D-mannitol solution using a trimmed 1 ml pipette tip; avoid taking plant materials away
6. Add enzyme solution from step 2 into the flask
    - As softly as possible using trimmed pipette tip
    - Make sure all leaf pieces are in the solution
7. Vacuum for ~45 min under dark

8. Shake (30-40 rpm; 25-26 °C; under dark) for ~5 hours
    - Check the results regularly
9. Filter the enzyme solution (containing protoplasts) through 40 μm nylon meshes
    - Shake the flask slowly before transfer (avoid the protoplasts sink at the bottom)
    - Using trimmed tips
    - Add W5 Solution, wash the flask first, and then transfer the solution onto the nylon meshes



10. Wash the protoplast using 21% Sucrose Solution (2.1 g sucrose in 10 ml water)
    - Add 5 ml 21% sucrose into a 15 ml round bottom falcon tube
    - Using trimmed tips, add protoplast solution (~ 5ml) from the petri dish softly on top of the 21% sucrose buffer; therefore, 4 tubes are needed as the volume of the protoplast solution is > 15 ml (15 ml enzyme solution + W5 solution)
    - Swirl the petri dish slowly when transferring protoplast solution (protoplasts are at the bottom); petri dish can be washed by W5 solution
11. Centrifuge at 250 g for 3 min
12. Take away the middle layer (containing protoplasts)
    - About 2 ml
    - When pipetting, move the trimmed tip around the tube
    - Before pipetting protoplasts, could take away part of the top layer (~ 2ml) first



13. Wash the protoplast using W5 solution
    - Add 3 ml W5 solution into a new 15 ml falcon tube
    - Add protoplasts solution (~2 ml) softly into the tube; mix gently and slowly
    - Add 2 ml W5 solution; mix gently and slowly
14. Centrifuge at 250 g for 3 min
15. Remove supernatant using trimmed tips
16. Wash the protoplasts again using W5 solution
    - Add 2 ml W5 solution into the tube; mix gently and slowly

- Add another 2 ml W5 solution; mix gently and slowly

17. Centrifuge at 100 g for 2 min
18. Remove supernatant using trimmed tips
19. Add 1 ml W5 solution, and put the tube on ice for 30 min (under light)
    - If using 4 tubes at step 10), mix two tubes into one at this step. Therefore, there will have two tubes, with 2 ml W5 solution in each one
20. Centrifuge at 100 g for 2 min
21. Remove the supernatant, and add 400 μl MMG Solution in each tube; mix gently and slowly
    - If there are 2 tubes, there will have 800 μl MMG, which would be enough for 3 transformations
22. For each transformation, in the 2 ml tube (follow the order below)
    - Add 30 μl plasmid (10 μg for each construct)
    - Add 200 μl MMG (containing protoplast), and mix
    - Add 230 μl fresh PEG-4000 Solution, mix gently and slowly
23. Put the 2 ml tube under dark for 15 min at room temperature
24. Wash by W5 solution
    - Add 460 μl (1X volume) W5 solution; mix gently and slowly
    - Add 920 μl (2X volume) W5 solution; mix gently and slowly
25. Centrifuge at 250 g for 3 min
26. Remove the supernatant
27. Add 400 μl W5 solution into each 2 ml tube; mix gently and slowly
28. Prepare the cell culture plate
    - Coat the plate with 5% BSA (~600 μl)
29. Transfer 400 μl W5 solution (containing protoplasts) into the well
30. Cover cell culture plate using foil

Solution

D-mannitol Solution

	For 10 ml	For 15 ml	Final concentration
D-mannitol	1.09 g	1.635 g	0.6 M
H_2O	9.5 ml	14.25 ml	

Enzyme Solution

Add in order	Working solution	Stock solution	For 50 ml	For 15 ml
1)	ddH_20		25 ml	7.5 ml
2)	0.4 M mannitol	1 M mannitol	20 ml	6 ml
3)	10 mM MES (pH 5.7; KOH)	0.5 M MES	1 ml	0.3 ml
	Preheat the solution at 70 °C for 3-5 min to activate the enzymes added later			
4)	1.5% Cellulase RS		0.75 g	0.225 g
5)	0.75% Macerozyme R-10		0.375 g	0.125 g
	Warm the solution at 50°C for 10 min to inactivate DNase and protease, and enhance enzyme solubility Cool the solution at 25 °C/RT; the final enzyme solution should be clear light brown			
	10 mM $CaCl_2$	1 M $CaCl_2$	500 µl	150 µl
	0.1% BSA	5% BSA	1 ml	300 µl
	20 mM KCl	0.5 M KCl	2 ml	600 µl
	5 mM β-mercaptoethanol	13.4 M ???	17.5 µl	5.6 µl
	Filter the final enzyme solution through a 0.45 µm syringe filter into a petri dish			

W5 Solution

	For 50 ml	Final concentration
MES (0.5 M; KOH, pH 5.7)	200 µl	2 mM
NaCl (1 M)	7.7 ml	154 mM
$CaCl_2$ (1 M)	6.25 ml	125 mM
KCl (0.5 M)	0.5 ml	5 mM
H_2O	~35.35 ml	
Store at room temperature		

MMG Solution

	For 10 ml	Final concentration
MES (0.5 M; KOH, pH 5.7)	80 µl	4 mM
Mannitol (1 M)	4 ml	0.4 M
$MgCl_2$ (1 M)	150 µl	15 mM
H_2O	Up to 10 ml	
Store at room temperature		

PEG-4000 Solution

	For 1 ml	For 5 ml	Final concentration
PEG-4000	0.4 g	2 g	
Mannitol (1 M)	400 µl	2 ml	0.4 M
$CaCl_2$ (1 M)	100 µl	0.5 ml	0.1 M
H_2O	140 µl	700 µl	