PREPARATION AND TRANSFECTION OF NICOTIANA MESOPHYLL PROTOPLASTS

Materials:

- Mannitol (Sigma M4125)
- MES hydrate (M8250)
- Polyethylene Glycol Mn 4000, platelets (Sigma, 81240)
- Cellulase R10(example: Duchefa C8001)
- Macerozyme R10 (example: Duchefa M8002)

Buffers and solutions:

1. **Stock solutions:**
   5M NaCl, 3M KCl, 1M $CaCl_2$, 0.5M Mannitol, 1M $MgCl_2$, 0.5M MES-KOH pH5.6.

2. **Digestion solution:**

1.5% cellulase
0.3% macerozyme
0.4M mannitol
20mM KCl
20mM MES pH 5.6
10mM CaCl2
0.1% BSA (added before use)
Total 50 ml for each solution
**The buffer can be prepared without the cellulose and macerozyme enzymes - these can be added immediately before starting the digest*

3. **Wash solution (originally W5):**

154mM NaCl
125mM CaCl2
5mM KCl
2mM MES pH5.6

4. **MMg solution:**

0.4M mannitol
15mM MgCl2
4 mM MES pH 5.6

5. **PEG solution:**

2g PEG 4000 Fluka
2 ml of 0.5M Mannitol
0.5ml of 1M CaCl2
Always prepare well in advance (at least 1hr) as the PEG takes a while to dissolve (manually rotating and warming will help).

STEPS IN PROCEDURE

I. Preparation of protoplasts from *Nicotiana* species.

Note: this procedure will prepare sufficient protoplasts for approximately 10-20 transfections

1. Grow plants in light intensity of 50-100 micmol/m-2/s-1 before preparing the protoplasts from them. Do not use biological control pesticides (such as Entonem

etc.). Do not use any chemicals or grow in conditions (e.g. irregular watering) that may cause stress to the plant.
2. Prepare 10ml digestion solution.
3. Bring young (4 week old) plants from the glasshouse/growth room to the lab immediately before the transformation.
4. Infiltrate the solution into the base of the leaves using a needless 1 mL syringe (see SOP for N. *benthamiana* infiltration). Infiltrate the entire leaf.
5. Cut the leaf into two pieces, remove the midrib, and if possible cut out the most conspicuous veins. Transfer the reserved leaf sections in the remaining enzymatic solution in a polystyrene dish.
6. Incubate up to 4 hours - wrap the polystyrene plate in paper and place in a controlled growth chamber. Occasionally (once an hour) gently shake the plate manually for several seconds. Usually *N. benthamiana* tissues will be satisfactorily digested in ~ 3 hour. *N tabacum* tissues will be digested in ~4 hours.
7. Filter through a 75 µm filter (for example BD cell strainer). Add 2 mL of W5 buffer, tilt gently to release additional protoplasts remaining in the tissue.
8. Transfer to a 50 mL plastic tube.
9. Centrifuge for 2 min at 100 rcf.
10. Discard the supernatant quickly with 10 mL pipette (without inverting the tube).
11. Re-suspend in 2 mL W5 buffer
12. Leave on ice for 30 minutes until the protoplasts precipitate by gravity.
13. Carefully remove the supernatant.
14. Re-suspend to a concentration of $2*10^{4-5}$ protoplasts/mL in MMg buffer.

II. Transfection with DNA

1. Add 10-50 µg of purified plasmid or linear DNA (1micg/micL, MaxiPrep Qiagen) to an Eppendorf tube.
2. Add 100 µL of protoplasts and mix well but gently by inverting the tube (avoid pipetting in every stage)
3. Slowly add 100 +x µL PEG solution (x = volume of the DNA added, for example add 110 µL of PEG if 10 µL of DNA was added) to the tube wall and mix well by tilting.
4. Incubate on the bench at RT for 2-5 minutes.
5. Add 600 µL W5 solution and gently mix by slowly inverting the tube.
6. Spin at 100g for 2 minutes (benchtop centrifuge)
7. Pipette out the supernatant very carefully - try not to remove any protoplasts.
8. Incubate the relevant wells in multi-well plate (Sterilin) with 0.1% BSA in DDW for several minutes. Discard the BSA solution from the multiwall plate.
9. Re-suspend in 300 µL of W5 buffer/reaction and leave overnight in a growth chamber in the BSA-coated multiwell plate. The Growth chamber should be set to 22^0C, light period of 18 hours, intensity of light should not exceed 70-100 micmol/m-2/s-1.
10. Protein expression can be detected after approx. 18 hours, and for at at least a further 24 hours.
11. For DNA preparation, scale-up protoplast transformation x2 (200 µL of protoplasts instead of 100 µL), collect the protoplasts from the multiwell plate, pellet the protoplasts at 100 rcf and discard the supernatant. Freeze the pellets or add CTAB or NaOH or denaturation reagent, as per the chosen DNA extraction method.