

Transient gene expression in moss protoplasts

For the rapid and quantitative analysis of promoter activity, transient expression provides an easily amenable tool. We use transfection of moss protoplasts to analyse the promoters of ABA-inducible genes. The protocol outlined below follows, for the most part, the protoplast isolation and DNA-delivery procedures we routinely use for stable transformation of moss protoplasts. However, transient expression assays take only 3 days to complete.

Whereas the isolation of stable transformants (targeted or otherwise) is best achieved using linearised DNA, transient expression experiments can be conducted using supercoiled plasmid DNA. In our experiments, we use promoter-GUS fusion constructs.

I. Protoplast isolation.

Solutions:

8.5% (w/v) mannitol: 8.5g D-mannitol dissolved in ddH₂O and made up to 100ml

8% (w/v) mannitol: 40g D-mannitol dissolved in ddH₂O, made up to 500ml and autoclaved.

40% PEG solution: 4g PEG-6000 dissolved in 10ml "MCT".
MCT = 9ml 8% mannitol + 1ml 1M Ca(NO₃)₂ + 0.1ml 1M Tris-Cl, pH 8. This solution is filter-sterilised and stored in 1 ml aliquots in sterile Eppendorf tubes at -20 C. – Do not re-freeze.

MMM solution: 910mg D-mannitol
0.15ml 1M MgCl₂
1ml 1% (w/v) MES-KOH, pH 5.6
ddH₂O to 10 ml
Filter-sterilised and stored in Sterilin tube at -20 C

Water: Double-distilled H₂O, sterilised by autoclaving.

PRML solution: 6% mannitol in BCD-TES-NH₄tartrate-10mM CaCl₂
Prepare as a stock lacking CaCl₂ and autoclave in 10ml aliquots. Add 0.1ml sterile 1M CaCl₂ immediately before use.

Materials (prepared and ready beforehand)

3 x sterile Universal bottles and 1 x sterile Bijou bottle and caps (Place in Magenta pot and sterilise).

2 x 100ml conical flasks, sealed with Al-foil.

Blue Gilson tips

Cutoff Blue Gilson tips (points truncated by clipping with scissors to produce wide bore)

Yellow Gilson tips

Short-form Pasteur pipettes (Sealed in glass boiling tube and autoclaved)
Plugged long-form Pasteur pipettes (Sealed in glass boiling tube and autoclaved)
(Plug these with cotton-wool)

Filter-Funnel-Conical: For filtering protoplasts - comprises a 100ml conical flask, a glass funnel containing a folded nylon mesh (100_μm) filter. The whole wrapped in Al-foil and autoclaved.

Sterile 1.5ml Eppendorf tubes (inside a Magenta pot or similar, and autoclaved)
Sterile round-bottom 2ml Eppendorf tubes (inside a Magenta pot or similar, and autoclaved)

Stainless steel spatula
Fine-tipped forceps
Gilson P-1000, P-200 and P-20 pipettes

Plastic 10 ml round-bottom tubes (Sterilin)

Test tube rack for Sterilin tubes, wrapped in Al-foil to keep tubes dark
Eppendorf tube rack

Procedure:

1. Enzyme

Weigh out 0.1g **Driselase** (Sigma D-9515, stored at -20 C) and mix with 10ml **8.5% mannitol**, in 10 ml Sterilin tube. Lay on bench at room temperature and gently agitate from time to time for a period of about 15 minutes. This looks like a cloudy brown suspension.

2. Digestion

Take the Driselase and all other requirements to the tissue culture room. Centrifuge the Driselase at 2,500 rpm for 5 minutes in the tissue culture room bench centrifuge, with the brake set on “high”.

The supernatant is a clear solution, with the appearance of weak tea. There is a substantial beige precipitate.

Transfer the supernatant by pouring it into the barrel of a 10ml syringe, fitted with a 0.45_μm sterilising unit, and push the solution through the filter into a sterile Universal bottle (flame the neck in a Bunsen flame).

Using an ethanol-dipped and extensively flamed (and cooled) spatula, scrape 6-day old moss homogenate culture from two cellophane-overlay plates (BCD-NH₄) and transfer the moss tissue to the Driselase solution. Cap the Universal bottle (flame the cap), seal this further with Parafilm and gently swirl.

Put the bottle in a Magenta pot half-full of water at 25 C as a makeshift water bath, to maintain an even temperature, and incubate with occasional GENTLE swirling for 1 hour.

3. Preparation

While the moss is being digested, prepare for the subsequent steps.

DNA: Plasmid DNA for transformation is good quality maxiprep DNA that has been ethanol-precipitated and dissolved in sterile dd H₂O. The concentration of the DNA stocks should previously have been accurately determined by both A₂₆₀ measurement and by agarose gel electrophoresis. The aim is to be able to deliver 8 µg plasmid DNA in a volume of **not more than 50 µl**.

Set up “n” Eppendorf tubes (where n= number of different transformations), each containing 8 µg plasmid DNA, and label these appropriately. Store these on ice.

PEG: Set up “n” 10ml Sterilin tubes, each containing 300 µl PEG solution, in a foil-wrapped tube rack (shields the protoplasts from the light). Label these appropriately. Also set out one more empty Sterilin tube for harvesting protoplasts.

8% Mannitol: Flame off the neck of a 100ml conical flask and pour in some 8% mannitol. You will need approximately 10ml per transformation.

MMM: Thaw the MMM stock. Set out “n” Eppendorf tubes in the tube rack. These will be used for aliquotting protoplasts.

4. Harvesting protoplasts:

Filtering: Unwrap the filter-funnel-flask, and wet the nylon filter with 8% mannitol. This prewetting is important to reduce surface tension when the moss digestion mixture is added to the funnel.

Transfer the digested moss tissue to the funnel, using a sterile plastic disposable 10ml pipette: pipette the suspension gently, to avoid shearing protoplasts, and aim to deliver the suspension over the entire area of the filter. The filter becomes clogged up as the funnel fills, so gently disperse the material by GENTLY sucking up and re-releasing the fluid, until all the liquid has filtered into the 100ml conical flask.

Centrifugation: Transfer the filtered protoplasts to the empty 10ml Sterilin tube, using a new 10ml plastic pipette. Dispense the liquid very gently to avoid bursting the protoplasts. Spin the tube in the bench centrifuge at 800 rpm for 5 minutes with the **brake OFF**.

(NB While this is spinning, wash out the filter and funnel, rinsing the filter thoroughly. If you don't do this, then the mannitol precipitates out as it dries and clogs the filter).

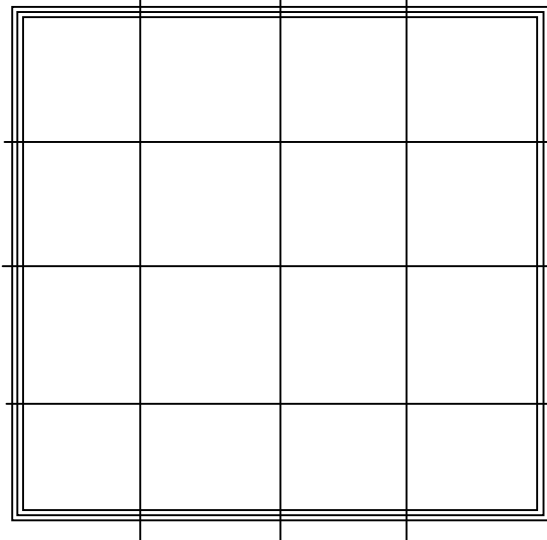
Wash No. 1: After the centrifugation, carefully remove the supernatant from the pellet (dark green) using the same 10ml pipette used to deliver the suspension to the tube in the first place. Be careful not to disturb the pellet, by leaving a little residual liquid above the pellet. Now VERY GENTLY loosen the pellet by slow and gentle rotation/tilting of the tube. When it looks like the pellet has resuspended, add 10ml 8% mannitol delivered by the same 10ml pipette. – Resuspension should be slow and gentle: suck up 10ml mannitol, and gently dribble 2ml onto the pellet at a rate of approximately 1ml per 10 seconds. Now, gently insert the tip of the pipette into the

partly resuspended protoplasts and suck them very slowly into the pipette. Release them slowly back into the tube. Repeat. Finally, gently deliver all the liquid in the pipette, by dribbling it down the wall of the tube from a point half-way up, slowly (1ml/10 sec) at first, then gradually more quickly. Recentrifuge at 800 rpm for 5 minutes with the brake off.

Wash No. 2: Repeat as above. This time, after adding all the mannitol, cap the tube and mix well by gentle rotation/tilting of the tube. When the protoplasts are completely resuspended, remove a small aliquot (about 0.1 ml) and put it in an Eppendorf tube. Centrifuge the tube as before (800rpm, 5 min, brake off).

Count protoplasts: While the protoplasts are being centrifuged, take the aliquot up to the lab to inspect and count them:

- Put a cover-slip on the haematocytometer (breathe on the slip and press into place, or moisten the edge of the slip with spit).
- Ensure the protoplast aliquot is properly resuspended by sucking up and down gently, with a short-form Pasteur pipette.
- Load the haematocytometer by placing a droplet on either side of the cover slip and letting the liquid enter the chamber.
- Examine with the Nikon microscope using the x10 objective. Count the protoplasts within the “triple-square” grids:



The region bounded by the triple lines has the volume $1 \times 1 \times 0.1 \text{ mm}^3$. Count the number of protoplasts in this. – To get reliable data, count the number in 8 of these, and determine the average. The final concentration of protoplasts/ml in the protoplast suspension is 10^4 times the average number.

For transformations, we use $1.6 \cdot 10^6$ protoplasts per ml, so to get this, just divide the average number by 16, and resuspend the protoplasts in that number of mls MMM solution.

(i.e. if the average no of protoplasts per triple-line square = 48, then they should be resuspended in $48/16 = 3$ ml MMM solution).

Resuspend protoplasts: When the protoplasts have spun down, carefully remove the supernatant using a clean 10ml pipette. The pellet is VERY sloppy, so be careful not to resuspend it and lose protoplasts! A small amount of residual liquid usually remains above the pellet. Now add the calculated volume of MMM solution, and gently disperse the protoplasts by sucking them gently up into a 10 ml pipette and transferring them into a sterile Universal bottle. Suck the suspension in and out of the pipette SLOWLY, a few times to ensure resuspension.

II. Transfection with DNA

1. DNA delivery

Set up “n” 1.5ml Eppendorf tubes, and carefully dispense 0.3ml aliquots of the protoplast suspension into each one, using a P-1000 and cutoff Blue tip.

Take the Eppendorfs containing the DNA from the ice bucket.

Loosen the caps on the labelled Sterilin tubes containing the PEG solution.

- Use a plugged, sterile, long-form Pasteur pipette to gently suck up the first aliquot of protoplasts. (Briefly flame the tip of the pipette, first)
- Mix it with the DNA solution, by gently pipetting it in and out of the DNA tube.
- Now transfer the mixture to the Sterilin tube, dispensing the mixture slowly and gently directly into the PEG solution, while simultaneously stirring the PEG solution with the tip of the Pasteur. Complete the mixing by gently sucking the mixture in and out of the pipette. Cap the tube.

Repeat this for all of the transfections.

Now place all the tubes in a wire rack, in a 45 C water bath and incubate them for 5 minutes (heat-shock). At the end of this time, remove the tubes and place them back into the foil-wrapped rack in the flow hood. Incubate them at room temperature for 10 minutes.

2. Dilution

Dilute the protoplasts by incremental addition of 8% mannitol over a 1 hour period.

First, carefully add 300 μ l to each tube, and mix very gently by rotating and tilting the tube. – Do not tilt more than is necessary to send the meniscus as far as the first line around the sterilin tube (the 5 ml mark).

5 minutes later, add a further 600 μ l 8% mannitol, and mix as before. This time, the meniscus can be tipped as far as the second (10ml) line.

5 minutes later, add a further 600 μ l 8% mannitol and mix as before.

Now continue, adding 1ml aliquots at regular intervals up to a final volume of 10 ml (= 6 additions of 1ml plus 1 addition of 1.4ml) over a period of 1 hour, start to finish (about 8-minute intervals). Mix well each time.

Note: At this point, the tubes can be left for a couple of hours: this is often a convenient time to break for lunch.

3. Recovery and incubation

Set up “n” x 2ml round-bottom Eppendorf tubes, to receive each transfected protoplast resuspension. Also set up sets of 4 x 1.5ml Eppendorf tubes in which the incubations will be performed. Two of these are for duplicate controls (no hormone), the other two for duplicate ABA treatments, so label these appropriately, with the name of the construct, and the treatment.

Spin down the diluted protoplasts at 800rpm for 5min, brake off.

Remove the supernatant from the first tube with a 10ml pipette, being very careful not to disturb the very sloppy protoplast pellet. Try not to leave too much residual liquid over it, although some must necessarily remain.

Immediately resuspend the pellet in 1ml PRML, added with a cutoff Blue tip from a P-1000. Transfer this into the appropriately labelled round-bottom, 2ml Eppendorf. – This can be done by sucking it up into the 10ml pipette for transfer, or by using the Gilson plus cutoff Blue tip. Immediately add another 1ml PRML and mix so that the protoplasts are evenly resuspended.

Using the cutoff Blue tip, dispense 0.5ml aliquots of the resuspended protoplasts to each of the 4 x 1.5ml incubation Eppendorfs.

Now add either 5 μ l sterile ddH₂O or 5 μ l 10⁻³M ABA to each tube (depending on treatment). Cap the tubes.

Repeat this procedure for each transfection.

Finally, put the tube rack containing the incubation mixtures in a light-tight cardboard box, and transfer this to the growth room. Incubate for 24 hours, and then remove the tube rack from the box and continue to incubate it under continuous illumination.

The following morning, the protoplasts are ready for harvesting and GUS assay.

III. GUS activity assay

This follows the general protocol established by Richard Jefferson.

1. Solutions

GUS buffer

50mM NaPO₄ buffer pH 7.

10mM Na₂EDTA

0.1% Triton X-100

5mM MSH (Added as 7ul neat MSH to 10 ml, immediately before use)

GUS assay buffer

GUS buffer containing 1mM methyl-umbelliferyl glucuronide (MUG)). Note: MUG is stored as a 100mM suspension in water, at -20 C. To dispense this, thaw the suspension and vortex vigorously. Then rapidly remove an aliquot using a circumcised yellow tip, for addition to the GUS buffer.

Stop solution

0.2M Na₂CO₃

Standard solution

100mM 4-methylumbelliferone in water. Stored at -20 C. This is used to prepare a range of standards for quantitating the reaction products.

2. Setup

First, set up the reaction mixtures and stop buffer tubes for the assay. For each reaction mixture (two replicates per treatment) set up 3 stop tubes labelled “0” (zero-time control), “30” and “60” (30-minute and 60-minute time-points). Into each stop-tube, dispense 450 ul 0.2M Na₂CO₃. (*i.e.* for each transfected protoplast treatment, you need 2 reaction tubes, 2 zero-time stop tubes, 2 thirty-minute stop tubes and 2 60 minute stop tubes = 8 tubes). Alternatively, instead of setting up 30 and 60 minute time-points, compromise with a single 40-minute time-point (corresponds to 2 x reaction, 2 x zero and 2 x 40-minute tubes = 6 tubes per reaction).

Into each reaction tube, dispense 150 ul GUS assay buffer .

Switch on the dri-block and equilibrate it at 37 C

When all the reaction and stop tubes are ready, and the dri-block is at a steady temperature, proceed with the assay:

3. Harvest protoplasts

At the end of the incubation time, harvest the protoplasts by spinning for 4 minutes at 1,000 – 2,000 rpm in the variable-speed Eppendorf microcentrifuge.

Remove the supernatant carefully with a drawn-out Pasteur pipette attached to a vacuum line

Lyse protoplasts in 160 ul GUS buffer (lacking MUG), by repeated squirting through a yellow Gilson tip.

Spin in microfuge at full speed, and transfer supernatant to a fresh tube, kept on ice.

Remove 50ul and transfer to a tube containing 450_1 acetone, and cap the tubes. Leave on ice.

4. Reactions

Start the reactions by adding 50ul protoplast lysate to a reaction tube. Immediately remove 50ul of the mixture and add it to the appropriate zero-time stop tube. Cap the reaction tube and place it in the dri-block to commence the incubation.

Repeat for a replicate sample. It is a good idea to stagger the setting up of the reaction tubes at 1-minute intervals, to ensure that all the reactions proceed for exactly the

same lengths of time. If you are VERY experienced, you may be able to reduce the interval between reactions to 30 seconds, but it is better to be on the safe side!!

Remove 50 ul aliquots from each reaction tube, and transfer these to the appropriate stop tubes at 30 minutes and 60 minutes after the start of the reaction. (Or 40 minutes if a single time-point is being used).

5. Fluorimetric measurement of reaction product

GUS reacts with MUG to release the fluorescent compound 4-methyl umbelliferone. A rapid check can be made on the course of the assay by placing the stop tubes on a UV transilluminator to observe the amount of fluorescence. This is also advisable in order to set up a series of standard solutions over the appropriate concentration range.

For the standards, dissolve 4-MU in water to prepare a 100mM solution. This can then be serially diluted in 0.2M Na₂CO₃, as appropriate. Prepare a range of standard solutions for which the highest concentration of MU generates fluorescence that appears just a bit brighter than the most intensely fluorescent of the enzyme assay stop solutions, when eyeballed on a transilluminator. – This is important, because fluorimeters generally work by scaling the readings according to the maximum fluorescence yield of a nominated sample. The precise procedure you follow will depend on the type of instrument available to you. In Leeds, we use the “Fluostar Optima”: an instrument that accepts 96-well plates, and for which it is possible to enter the concentrations of all the standards, so that the fluorimeter’s software will generate a standard curve and calculate the concentration of MU generated in all the experimental samples.

(For guidance, for the highly active wheat and moss “Em” promoters, the range of standard concentrations we use is from 0.1 to 50 μ M)

Results can be calculated as nmol 4-MU generated per minute. For each batch of protoplasts, this should be normalised for differences in the quantity of lysate added to each reaction mixture. Because the protoplasts are derived from a green tissue, we find the most convenient normalisation factor is a “per milligram chlorophyll” basis.

6. Chlorophyll determination

Chlorophyll determination is based on the 50 μ l aliquot mixed with acetone:

- Centrifuge this at 12,000 x g in a microcentrifuge and transfer the supernatant to a fresh tube.
- For each supernatant, measure the absorbance of the supernatant in a spectrophotometer at 645nm and again at 663nm (use acetone as a blank)

(Note: because acetone attacks most plasticware used in spectrophotometers, you will have to use glass cuvettes, or an appropriately resistant microplate).

Chlorophyll concentration is calculated according to the formula:

$$\text{Mg/ml chlorophyll} = [(8.02 \times A_{663}) + (20.2 \times A_{645})]/10$$