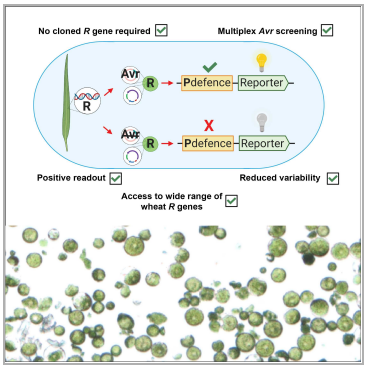

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Protocol status: Working
We use this protocol and it's working

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Wheat Protoplast Preparation and Transformation

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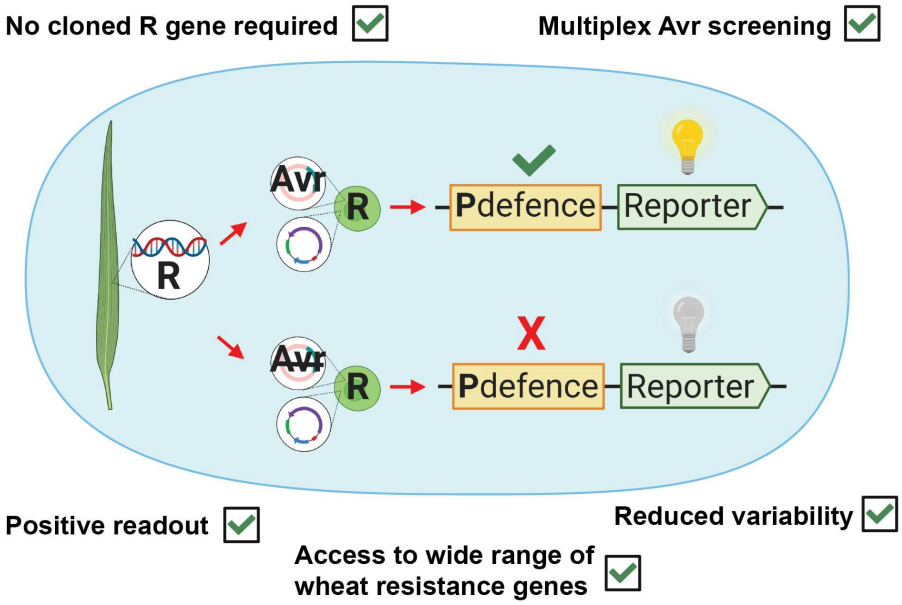
ABSTRACT

Wheat protoplast protocol for studying effector recognition and cell-autonomous defense signaling. Plant resistance (R) proteins recognize transiently expressed Avirulence *(Avr)* effector gene products, triggering defense responses. A co-transfected reporter plasmid enables quantification of response, upon activation of a defense-related promoter and expression of a luciferase reporter cassette.

This protocol contains instructions for plant growth, protoplast isolation, transfection using the PEG-Ca method, luminescence measurement via plate reader and data processing.



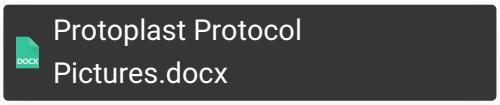
Wheat protoplast assay, showing wheat leaf carrying *R* gene, transfected with *Avr* and reporter constructs. Top track shows recognition and induction of plant defense response, leading to expression of reporter. Lower track shows no recognition, thus no defense response and low luminescence output from reporter.

IMAGE ATTRIBUTION

All images - Salome Wilson. Schematic created with BioRender

GUIDELINES

Some images of what to expect:



Timeline:
Plant Growth: 7-9 days (depending on cultivar)
Isolation: 90 mins
Incubation: 3 hrs
Washing: 1 hr
Transfection: 45 mins
Incubation: 4 hrs to overnight (16-18 hrs)
Luciferase Assay Plate read: 1hr
Data Processing 20 mins

MATERIALS

0.45 µm filter sterilise and store at RT:

- MES-KOH 0.2 M (Sigma M8250) adjusted to pH 5.7 with KOH
- Mannitol, 0.8 M (Sigma M8250)
- Mannitol 0.6 M (Sigma M8250)
- $MgCl_2$, 2 M (Sigma M2670)
- $CaCl_2$, 2 M (Sigma C7902)
- KCl, 2 M (Sigma P9541)
- NaCl, 2 M (Univar 465)

Store at 4ºC:

- Cellulase 'Onozuka' R-10 or Cellulase 'Onozuka' RS (Yakult)
- Macerozyme R - 10 (Yakult)

Store at RT:

- PEG 4000 (Sigma 81240)
- Wheat seeds, potting mix and fertiliser

Filter sterilise aliquots of required quantity and store at -20ºC:

- BSA 10% w/v (Calbiochem 126609)
- BSA 5% w/v (Calbiochem 126609)
- Purified plasmid DNA which will express reporter (GFP, LUC etc.)

Equipment:

- Syringe sterilisation filters 0.45 µm
- Cell strainer 100 µm
- 30 mL round-bottom tubes (Sarstedt 55.517)
- Small funnel

- Hemocytometer
- 12-well cell culture plates, clear (Sarstedt 83.3921.500)
- 96 well white opaque plates (for luminescence readings)

W5

Stored at RT, kept sterile. All components filter sterilised using a 0.45 um syringe filter

	Volume	Final Concentration
MES-KOH (0.2M, adjusted to pH 5.7 with KOH)	4 mL	2mM
KCl (2M)	1 mL	5mM
CaCl2 (2M)	25 mL	125mM
NaCl (2M)	30.8 mL	154mM
Sterile H2O	to 400 mL	

MMG

Stored at RT, kept sterile. All components filter sterilised using a 0.45 um syringe filter

	Volume	Final Concentration
MES-KOH (0.2M, adjusted to pH 5.7 with KOH)	0.8 mL	4mM
Mannitol (0.8M)	20 mL	0.4 M
MgCl2 (2 M)	0.3 mL	15 mM
Sterile H2O	to 40 mL	

W5 and MMG can be prepared in advance, enzyme solution and PEG-Ca should be made on the day of use.

Protoplast Stock Solutions .xlsx

Plant Growth

1 1w

1. ¾ fill a 10cm diameter pot with medium-grain potting mix without fertiliser
2. Sprinkle 10 to 20 seeds on top and fill pot to just below rim
3. Move to growth cabinet set at 21°C with 16 hr days
4. Place pots in shallow black tray and dampen mix with water mixed with complete soluble

fertiliser (Hortico brand, complete mix – 1 scoop/1L)

5. Add the fertiliser water to the tray, filling to approx 3cm below rim.
6. Check the plant at day 5 and top-up with plain water. Keep well-watered as health of plant is essential to protoplast success.
7. Plants are ready to use after 7-9 days (2nd leaf just emerging) Some wheat cultivars may emerge more slowly so adjust as necessary

Make enzyme solution

2 Prepare fresh on day of use & don't reuse:

	Volume	Directions	Final Concentration
MES-KOH (0.2 M)	0.5 mL		20 mM
Mannitol (0.8 M)	3.75. mL		0.6 M
KCl (2 M)	25 µL		10 mM
		55ºC for 5 min (water bath)	
Cellulase 'Onozuka' R-10 or RS	75 mg		1.5 % w/v
Macerozyme R-10	37.5 mg		0.75 % w/v
		55ºC for 10 min	
		cool to RT	
CaCl2 (2 M)	25 µL		10 mM
BSA (10% w/v)	50 µL		0.1 % w/v
H2O (Milli-Q)	to 5 mL	mix gently	
		filter 0.45 µm to ensure enzyme is completely dissolved	

Isolate protoplasts

3

1. Add 10 mL of 0.6 M Mannitol (filter sterilised) to a 7 cm petri-dish
2. Using a blunt razor, make a shallow cut in the abaxial or adaxial epidermis, and carefully peel epidermal layer off.
3. Place the leaf, peeled-side down in the Mannitol for 10 mins (any more than 20 mins and the leaves get too floppy) – try to fill the petri dish with as many leaves as possible, but be careful not to overlap them
4. Add 10 mL of the enzyme solution (freshly made) to another 7 cm petri-dish
5. Transfer the leaf peels from the mannitol to the enzyme solution using tweezers – being

careful to touch them as little as possible

6. Wrap the petri-dish in foil and incubate in the dark at room temperature with gentle rotation on orbital shaker (horizontal movement at 60 RPM) for three hours. Gently tap/swirl dish to release protoplasts, leaves will appear transparent.

During incubation

4

1. Prepare PEG-Ca solution (shake vigorously to dissolve PEG) Make freah each day, don't reuse

		Volume	Final Concentration
	PEG 4000	1200 mg	40 % w/v
	Mannitol (0.8 M)	750 µL	0.2 M
	CaCls (2 M)	150 µL	100 mM
	H2O	to 3 mL	

1. Dispense amount of W5 and PEG required for transfection according to setup
2. Thaw 5% BSA and label culture plates for incubation

4.1 **Prepare Plasmid DNA for Transfection step:**

1. Set up number of round-bottom tubes required according to setup sheet
2. Thaw plasmid DNA, flick and spin tubes to ensure homogenous mixture. Combine aliquots where needed to avoid variation between plasmid DNA batches. Freeze/thaw each aliquot three times only.
3. If plasmid appears viscous, heat at 65^{o}C for 10 min. This is due to high concentration of plasmid DNA (this may also affect concentration reading).
4. Dispense required amount into bottom of tubes (usually 20 µg reporter plasmid and 10-15 µg of Avr/R plasmid). Use a new tip each time to prevent cross-contamination.

Washing

5

1. Add 5 mL of W5 solution to petri dish
2. After three hours, remove the leaves from the enzyme solution using tweezers, gently lift up and down to release trapped protoplasts
3. Place a cell strainer in the lid of the petri-dish, add 1 mL of W5 to wet the strainer, then filter the protoplasts solution (make sure not to drop them from a height)
4. Using a funnel, transfer the filtered protoplasts to a 30 mL plastic, round bottomed tube, and add another 5 mL of W5
5. Gently swirl the protoplasts to mix, until just homogenous
6. Pellet at 100 x g for 3 mins
7. Carefully remove the supernatant (make sure not to suck up any of the protoplasts, and if you do, don’t pipette them back into the solution – once they’re gone, they’re gone, pipetting will destroy them)

8. Add 15 mL of W5
9. Estimate the concentration using a haemocytometer (at this stage there may be some chloroplasts/debris visible still)
10. Calculation: total number of cells = (average cells of 4 squares counted) x 10,000 x initial volume x dilution factor (Recommended: 5 µL protoplasts and 20 µL W5, (4x dilution) add 8 µL to each side of the haemocytometer)
11. incubate on ice in the dark for 40 mins or until the protoplasts have settled to the bottom of the tube (up to 1 hr)
12. Remove the supernatant, and adjust to 300,000-350,000 protoplasts/mL using MMG

Protoplasts are now ready for the transformation step (they should be used ASAP)

Transfection

6 1. Choose either option A for manual pipette or option B for electronic multichannel pipette (e.g. Integra Viaflo Voyager 8 tip 1250 µL pipette with adjustable spacing)

6.1 Transfection: Option A, manual pipette

1. Make sure all the PEG solution is dissolved
2. Pre-load a pipette with 300 µL of PEG (use wide bore tips or cut tips for all protoplast handling steps)
3. Gently swirl the protoplasts to resuspend, and make sure the suspension is homogenous - protoplasts will settle to the bottom of the tube very quickly
4. Add 300 µL of protoplasts to the DNA (in prepared tubes), swirl to mix and quickly add the 300 µL of PEG. Gently swirl to homogenise (PEG is highly viscous so be sure there is no clear layer at bottom of tube)
5. Repeat for all transformations, and incubate for 15 minutes
6. Add 900 µL of W5 to stop the transformation
7. Pellet the protoplasts at 100 x g for 2 mins
8. Remove as much of the supernatant as possible, without losing any of the protoplasts (it's better to keep the protoplasts than get all of the supernatant)
9. Coat the wells of a sterile 12 or 24 well cell culture plate with 5% BSA (filter sterilised) by adding 1 mL to the first well, and transferring to the next, leaving a coating behind
10. Resuspend in 500 µL of modified W5 (W5 + 1 mM glucose), then add to the culture plate using wide bore tips (or cut tips)

Incubate for 4 to 16 hours (depending on reporter expression) in light, at room temp

6.2 Transfection: Option B, multichannel electronic pipette

1. Setup prepared plasmid DNA in tubes in groups of 8. Alignment can be tricky as pipette is on maximum spacing, so manually move them to the same side of each well. Alternatively, use every 2nd tip to accommodate larger tubes.

2. Dispense protoplasts, PEG and W5 into reservoirs (add 10% extra volume, 1 extra rep per 10 samples). Protoplasts will settle very quickly so agitate gently by lifting one side of the reservoir before pipetting
3. Set electronic pipette to 'protoplasts300' program as follows:
4. Pipette 300 µL (protoplasts) and mix immediately. Eject tips.
5. Pipette 300 µL (PEG) and mix immediately (Pipette up and down 3x slowly)
6. Pipette according to program. Check tubes are mixed thoroughly.
7. Incubate 15 mins
8. Add 900 µL W5 to stop reaction
9. Pellet the protoplasts at 100 x g for 2 mins
10. Remove as much of the supernatant as possible with a manual pipette, without losing any of the protoplasts (it's better to keep the protoplasts than get all of the supernatant)
11. Coat the wells of a sterile 12 or 24 well cell culture plate with 5% BSA (filter sterilised) by adding 1 mL to the first well, and transferring to the next, leaving a coating behind
12. Resuspend protoplasts in 500 µL of modified W5 (W5 + 1 mM glucose), then add to the culture plate by manual pipette.

Incubate for 4 to 16 hours (depending on reporter expression) in light, at room temp

Luminescence Measurement

7

1. Transfer well contents to 2 mL tubes
2. Centrifuge 300 x g for 3 min at RT
3. Add 200 µL 1x lysis buffer to each tube
4. Vortex, incubate for 15 min
5. While waiting, prepare PCR tube strip of Luciferase assay substrate for multi-channel pipette
6. Centrifuge 300 x g for 3 min at RT
7. Add 50 µL lysate into white, opaque bottom 96 well plate

Go to plate reader for final addition of substrate:

1. Set up plate reader for dual luciferase reading, check that white, opaque bottom plate type is selected (e.g. Corning flat white)
2. Add 50 µL luciferin (Promega Steady-Glo) with multichannel pipette - pipette in/out 2 times to mix
3. Incubate 5 mins
4. Read using luminometer (1000 ms integration, 0 ms settle)

For ratiometric assay: (normalisation of ELuc readings with pRedF) select red and green filters to run immediately following total luminecesence measurement. Some plate readers have presets for the Chroma-Glo™ Luciferase Assay System. Otherwise select green and red filters.

Data Processing

8

Perform deconvolution calculations using measurements from red and green filters, using

spreadsheet template or Python script (also includes plotting). Note that deconvolution constants may vary between plate readers, requiring calibration measurements as described in Promega Chroma-Glo™ Luciferase Assay System Technical Manual (page 9).

Spreadsheet template for deconvolution calculations

Protoplast luminescence deconvolution and plotting.docx

Template for results input

Results_Template.xlsx

Instructions for plotting script

RatiometricAssayDeconvolutionCalculations.csv

Script

ADA_plotting_script.py