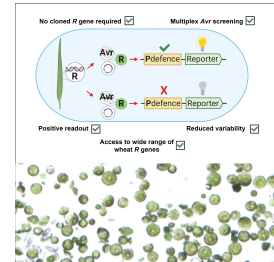


Apr 27, 2023

Wheat Protoplast Preparation and Transformation

DOI

<https://dx.doi.org/10.17504/protocols.io.q26g7r3zkvwz/v1>



Salome Wilson¹, Bayantes Dagvadorj¹, Rita Tam¹, Benjamin Schwessinger¹

¹Australian National University

Team Schwessinger



Salome Wilson

Australian National University

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.q26g7r3zkvwz/v1>

Protocol Citation: Salome Wilson, Bayantes Dagvadorj, Rita Tam, Benjamin Schwessinger 2023. Wheat Protoplast Preparation and Transformation. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.q26g7r3zkvwz/v1>

License: This is an open access protocol distributed under the terms of the **Creative Commons Attribution License**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

We use this protocol and it's working

Created: February 08, 2019

Last Modified: April 27, 2023

Protocol Integer ID: 20179

Keywords: wheat, protoplast, transient gene expression, rust fungi, transformation wheat protoplast protocol, wheat protoplast preparation, plant resistance, protoplast isolation, effector gene product, instructions for plant growth, luciferase reporter cassette, plant growth, gene, related promoter, protein

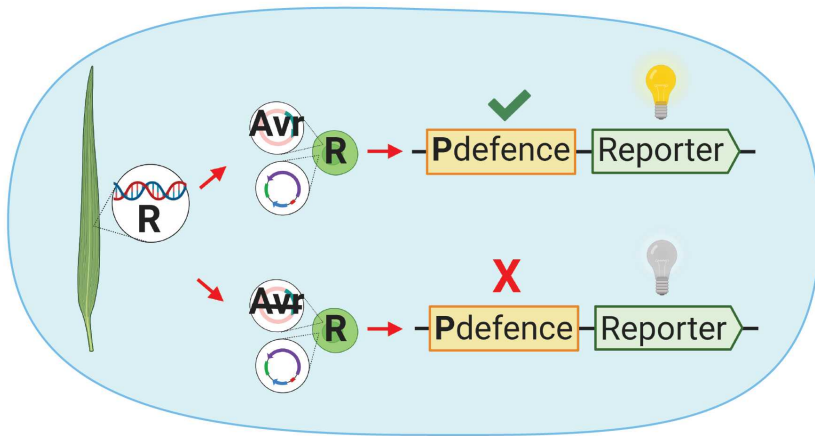
Abstract

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.

No cloned R gene required ☒

Multiplex Avr screening ☒



Positive readout ☒

Reduced variability ☒

Access to wide range of wheat resistance genes ☒

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:



Protoplast Protocol Pictures.docx

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)
- MgCl₂, 2 M (Sigma M2670)
- CaCl₂, 2 M (Sigma C7902)
- KCl, 2 M (Sigma P9541)
- NaCl, 2 M (Univar 465)

Store at 4°C:

- 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 -20°C:

- 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 µm syringe filter

		Volume	Final Concentration
	MES-KOH (0.2M, adjusted to pH 5.7 with KOH)	4 mL	2mM



	KCl (2M)	1 mL	5mM
	CaCl ₂ (2M)	25 mL	125mM
	NaCl (2M)	30.8 mL	154mM
	Sterile H ₂ O	to 400 mL	

MMG

Stored at RT, kept sterile. All components filter sterilised using a 0.45 µm 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
	MgCl ₂ (2 M)	0.3 mL	15 mM
	Sterile H ₂ O	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. $\frac{3}{4}$ 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

1w

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 μ L		10 mM
			55°C 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
			55°C for 10 min	
			cool to RT	
	CaCl ₂ (2 M)	25 μ L		10 mM
	BSA (10% w/v)	50 μ L		0.1 % w/v
	H ₂ O (Milli-Q)	to 5 mL	mix gently	
			filter 0.45 μ m 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 fresh each day, don't reuse

	Volume	Final Concentration
PEG 4000	1200 mg	40 % w/v
Mannitol (0.8 M)	750 µL	0.2 M
CaCl ₂ (2 M)	150 µL	100 mM
H ₂ O	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°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 μ L 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 μ L 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 μ L (protoplasts) and mix immediately. Eject tips.
5. Pipette 300 μ L (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 μ L 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 μ L 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 μ L 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 μ L 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 μ L 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 luminescence 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 deconvol...

Template for results input

 Results_Template.xlsx

Instructions for plotting script

 RatiometricAssayDeconvolutionCal...

Script

 ADA_plotting_script.py



Protocol references

Arndell, T., Sharma, N., Langridge, P. et al. gRNA validation for wheat genome editing with the CRISPR-Cas9 system. BMC Biotechnol 19, 71 (2019). <https://doi.org/10.1186/s12896-019-0565-z>

González-Grandío E, Demirer GS, Ma W, Brady S, Landry MP. A Ratiometric Dual Color Luciferase Reporter for Fast Characterization of Transcriptional Regulatory Elements in Plants. ACS Synth Biol. 2021 Oct 15;10(10):2763-2766. doi: 10.1021/acssynbio.1c00248. Epub 2021 Sep 14. PMID: 34520169.

Saur IML, Bauer S, Lu X, Schulze-Lefert P. A cell death assay in barley and wheat protoplasts for identification and validation of matching pathogen AVR effector and plant NLR immune receptors. Plant Methods. 2019 Oct 24;15:118. doi: 10.1186/s13007-019-0502-0. PMID: 31666804; PMCID: PMC6813131.