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Identifying direct TARGETs of transcription factors in wheat V.2

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Abstract

Transcription factors (TFs) are key regulators of expression of numerous genes. In wheat, many gene networks remain unresolved with the identification of TF target genes being particularly problematic. Methods developed for this purpose are not yet adequate. For example, ChIP-seq requires the use of transgenic plants which is still a lengthy process in wheat, while *in vitro* techniques such as DAP-seq are not effective on all families of TFs. To overcome these limitations, we have adapted the *Arabidopsis thaliana* TARGET system (Transient Assay Reporting Genome-wide Effects of Transcription factors) developed by Bargmann *et al.,* 2013[1], to wheat. This *in vivo* system uses transformed protoplasts for the rapid, genome-wide identification of the direct targets of TFs.

Materials

1) BUFFERS

NB:
- All buffers are filter sterilised (22 µm filter) once made, except the PEG solution (as it is too viscous).
- Cellulase RS, Macerozyme R10 and PEG 4000 listed here are important for the success of the isolation and transformation of wheat protoplasts. Alternative choices may result in lower transformation success rates.
- MES-KOH 100 mM, Mannitol 1 M and Plasmolysis buffer can be made the day before protoplast isolation.
- Enzyme solution needs to be made fresh on the day of protoplast isolation.
- MaMg solution, W5 solution and PIM can be made during the 4 hour enzyme incubation on the day of protoplast isolation.

MES-KOH 100 mM (50 ml – pH 5.6):
0.976 g MES
pH to 5.6 with ~1 ml KOH 1 M

Mannitol 1 M (50 ml): Create multiple stocks and freeze until needed.
9.11 g Mannitol
Heat to 55°C to dissolve.

Plasmolysis buffer (50 ml):
6.83 g Mannitol (final concentration 750 mM)
NB: May need heating to 55°C to dissolve.
50 µl $CaCl_2$ 1 M (final concentration 1 mM)
2.5 ml MES-KOH 100 mM (final concentration 15 mM)

Enzyme solution (10 ml):
0.15 g Cellulase RS (final concentration 1.5%) Duchefa Biochemie catalog number C8003
0.05 g Macerozyme R10 (final concentration 0.5%) Duchefa Biochemie catalog number M8002
2 ml H_2O
6 ml Mannitol 1 M (final concentration 0.6 M)
1 ml MES-KOH 100 mM (final concentration 10 mM)
Incubate for 10 minutes at 55°C to inactivate proteases and enhance enzyme solubility.
Once cool, add:
10 µl $CaCl_2$ 1 M (final concentration 1 mM)
1 ml 10 mg/ml BSA kept in 1 ml aliquots at -20°C (BSA final concentration 0.1%)

W5 solution (50 ml):
40.5 ml H_2O
6.25 ml $CaCl_2$ 1 M (final concentration 125 mM)
1.54 ml NaCl 5 M (final concentration 154 mM)

1 ml MES-KOH 100 mM (final concentration 2 mM)
0.25 ml KCl 1 M (final concentration 5 mM)

MaMg solution (10 ml):
3.3 ml H_2O
6 ml Mannitol 1 M (final concentration 0.6 M)
75 µl $MgCl_2$ 1 M (final concentration 15 mM)
400 µl MES-KOH 100 mM (final concentration 4 mM)

PEG solution – Make 15 min before use, long vortex to dissolve – DO NOT HEAT
1g POLY(ETHYLENE GLYCOL), AVERAGE MN 4,000 Merck (catalog number 81240)
375 µl H_2O
1 ml Mannitol 1 M
250 µl $Ca(NO_3)_2$ 1 M

Protoplast Incubation Medium (PIM - 50 ml)
30 ml Mannitol 1 M (final concentration 0.6 M)
2 ml MES-KOH 100 mM (final concentration 4 mM)
200 µl KCL 1 M (final concentration 4 mM)
1.8 ml H_2O
150 µl $CaCl_2$ 1 M (final concentration 3 mM)
H_2O to 50 ml

Dexamethasone (DEX) stock solution:
Dexamethasone bioreagent from Merck (catalog number D4902)
10 mM DEX stock dissolved in 100% ethanol and stored in aliquots at -20°C

Cycloheximide (CHX) stock solution:
Cycloheximide solution from Merck (catalog number C4859)
100 mg/ml (MW = 281.35 g/mol so 355 mM) CHX stock in dimethylsulfoxide stored in the fridge (NB: allow CHX stock to warm to room temperature before making the working solution on the treatment day, as it solidifies in the fridge)

2) MATERIALS

- MidiPrep kit (e.g. Qiagen Plasmid Midi Kit catalog number 12143)
- 2 litre pot, compost, tray and box to create a dark environment
- Scissors and single-edge razor blades
- 100 mm Petri dishes
- Aluminium foil (to create darkness)
- Sterile beaker (100 ml)

- Sterile 40 µm nylon mesh (e.g. VWR®, Cell Strainers catalog number 732-2757)
- Sterile 0.22 µm filters and plastic syringes (10 ml and 50 ml)
- 50 ml round-bottom centrifuge tubes (e.g. Falcon tubes)
- 2 ml Eppendorf tubes
- Hemacytometer
- FACS collection tubes (5 ml)
- RNeasy Micro kit from Qiagen (catalog number 74004)

3) EQUIPMENT

- Vacuum pump and desiccator
- Incubator/shaker (25°C, 40 rpm)
- Large centrifuge (for 50 ml tubes) which has slow acceleration & deceleration
- Tabletop centrifuge (for 2 ml Eppendorf tubes) which has slow acceleration & deceleration
- Vortex
- Epifluorescence microscope (to visualise bright field and GFP/RFP)
- FACS Discover S8 or equivalent (nozzle needs to be at least 130 µm)

Troubleshooting

Cloning of Transcription Factor (TF) of interest in TARGET plasmid via Gateway

1 Use Gateway cloning technology to clone a TF of interest in one of the TARGET plasmids (Brooks *et al.*, 2023)[2].
NB: it is possible to carry out this protocol for two TFs simultaneously if one is cloned in a GFP tagged plasmid and the other in an RFP tagged plasmid. Then GFP (TF1) and RFP (TF2) transformed protoplasts can be sorted separately via FACS as described in Brooks *et al.*, 2019[3].

2 It is recommended to check the correct construct assembly by sending it for sequencing before the next step.

3 Once the TF has been cloned in the TARGET plasmid and verified by sequencing, carry out a midiprep in order to get high concentration of construct (minimum 1 µg/µl). The construct will be used in the protoplast transformation step.

Protoplast isolation

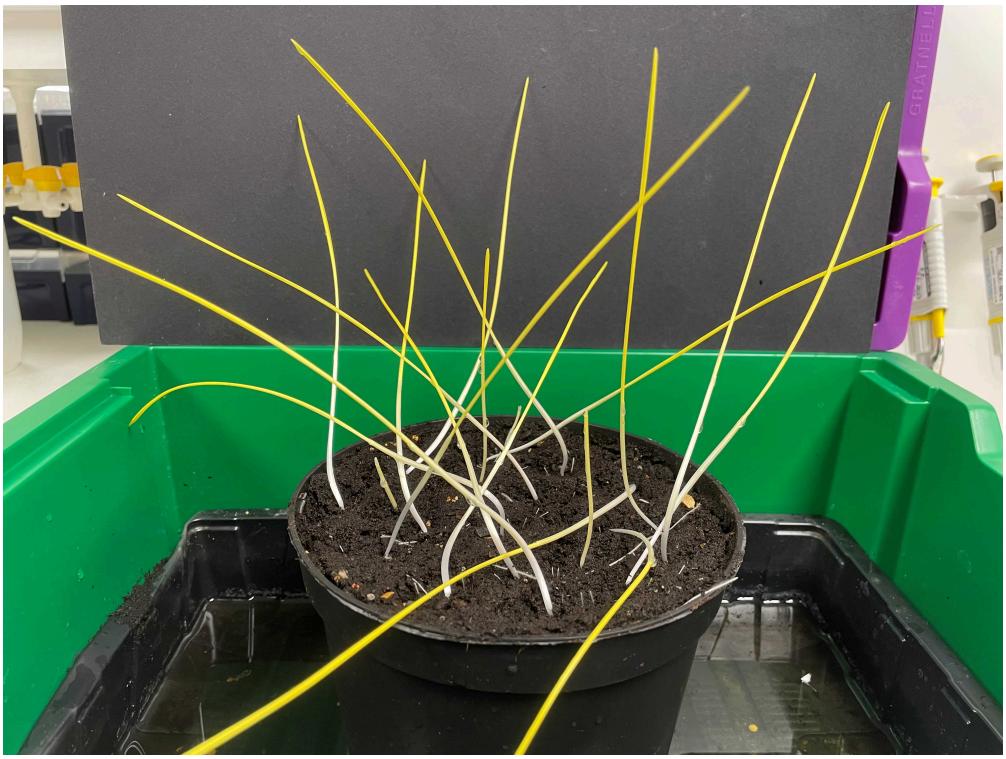
4 Sow 20 seeds of wheat (cultivar *Fielder*) in a 2 L pot containing a starter compost (e.g. 90% peat, 10% grit, 4 kg/m^3 dolomitic limestone, 1.2 kg/m^3 osmocote start). Water well and place the pot in a tray containing a layer of water (enough to keep the soil damp for 8-9 days). Place the tray in a box tall enough to allow seedlings to grow upwards, and if needed, surround the box with a black bag to ensure complete darkness. Leave the seeds to germinate and grow at room temperature (circa 23°C) undisturbed for 8 to 9 days.
NB: Cultivar *Fielder* is recommended as it achieves high protoplast transformation efficiency.

5 Switch on a water bath and heat up to 55°C. Thaw 1 M Mannitol at 55°C.

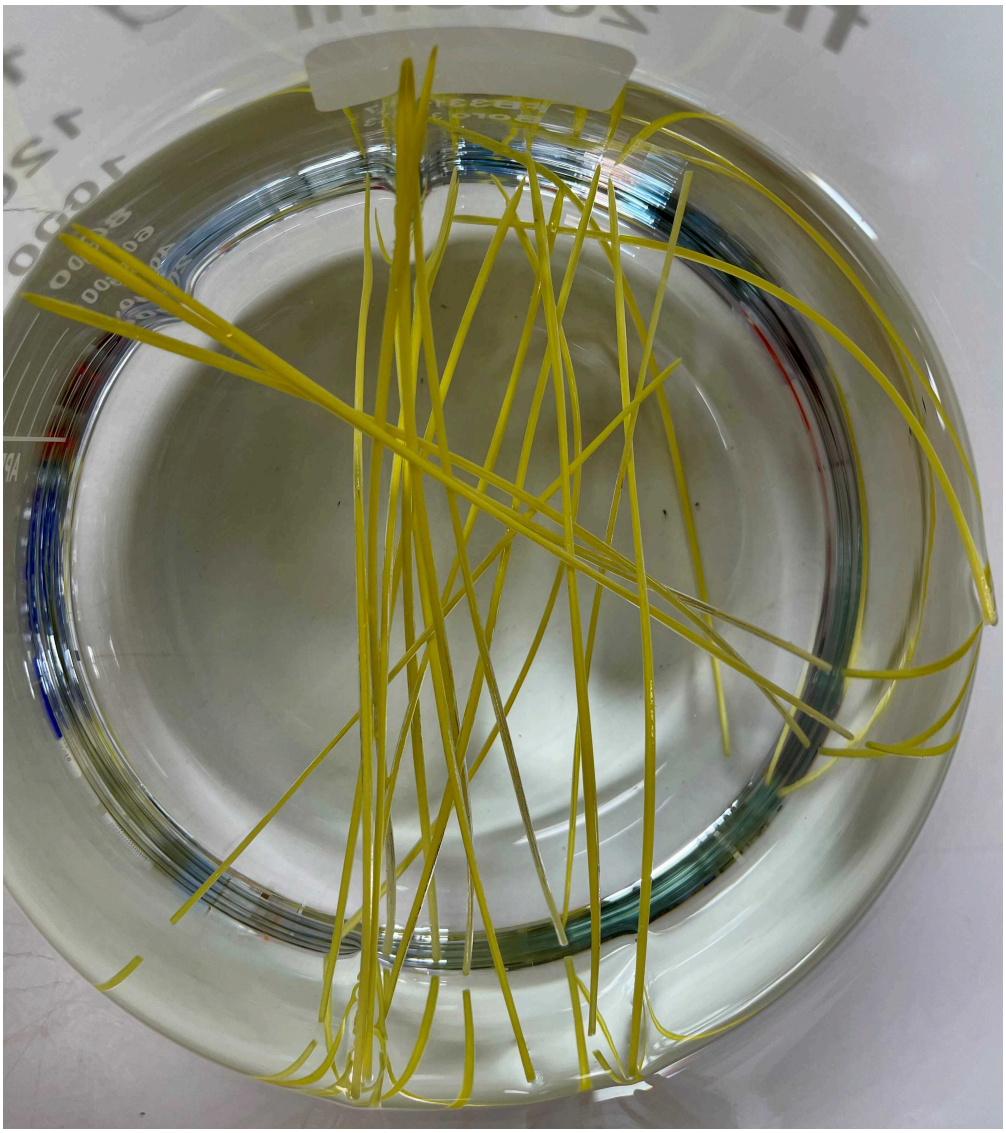
6 Make up fresh enzyme solution.

7 Cut 15 to 20 young leaves from 8-9 day old plants using clean scissors and swirl the leaves in distilled H_2O in a large beaker:

Cut just above the coleoptile (white) to ensure that only leaf material (yellow) gets harvested.



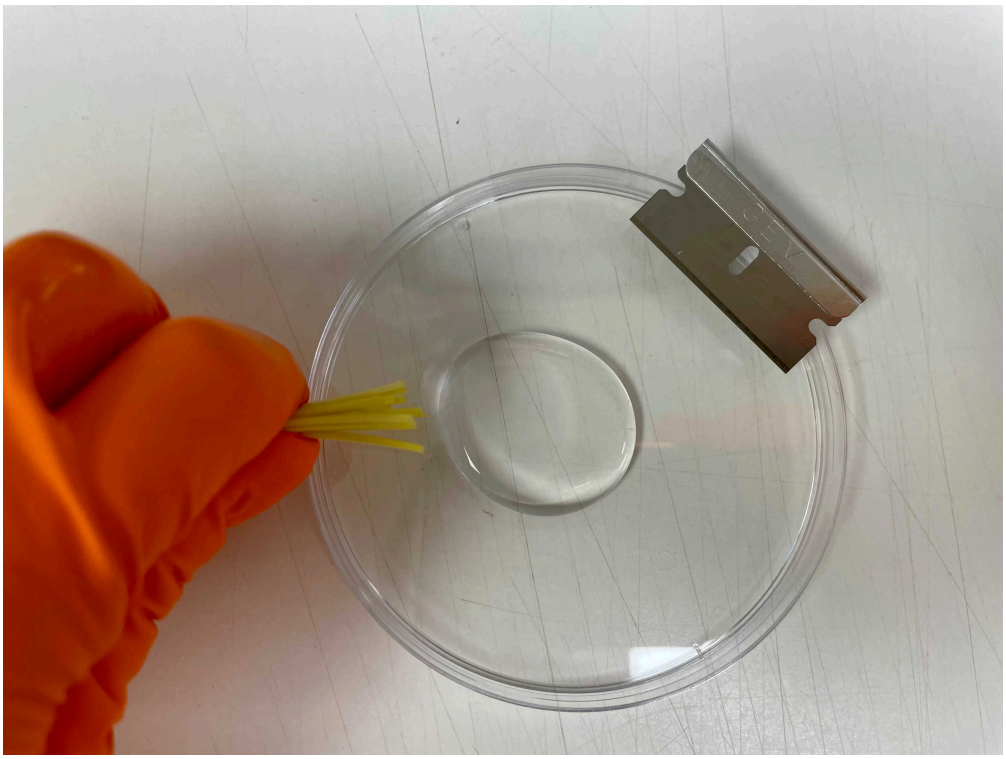
Wheat seedlings cv. *Fielder* after 8 days of growth in the dark.



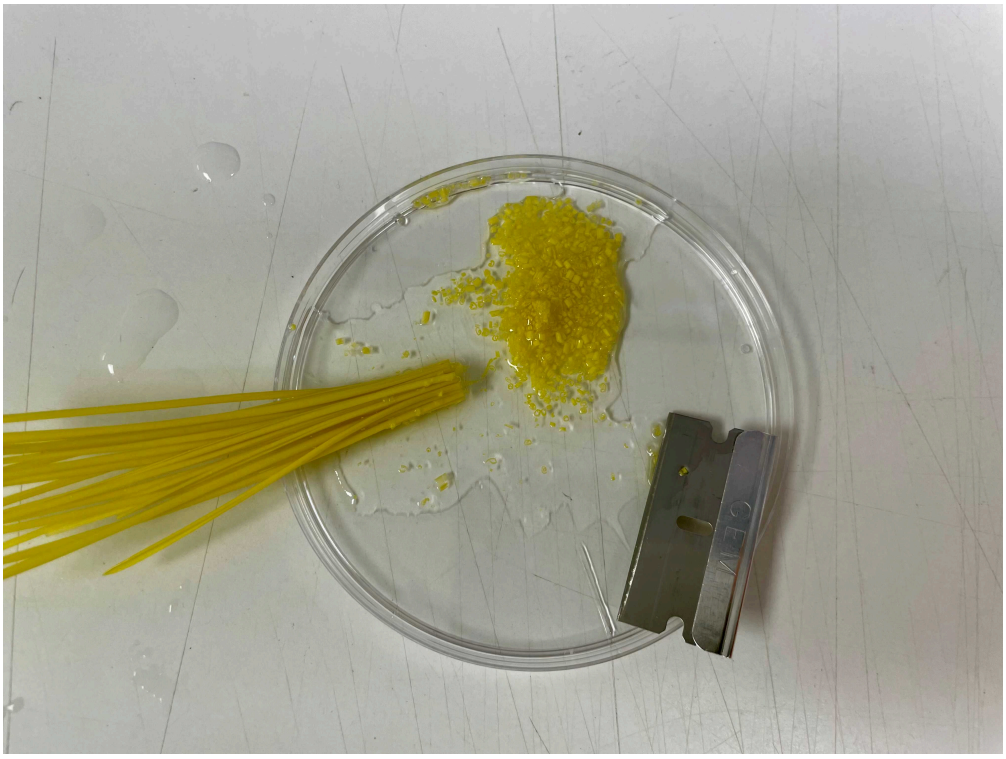
Cut leaves being swirled around in distilled H_2O.

8 Pour 1 ml of plasmolysis buffer into a Petri dish lid.

9 Bunch up the leaves, hold them in liquid in the Petri dish lid and cut them into 0.5 - 1 mm strips using a fresh razor blade.



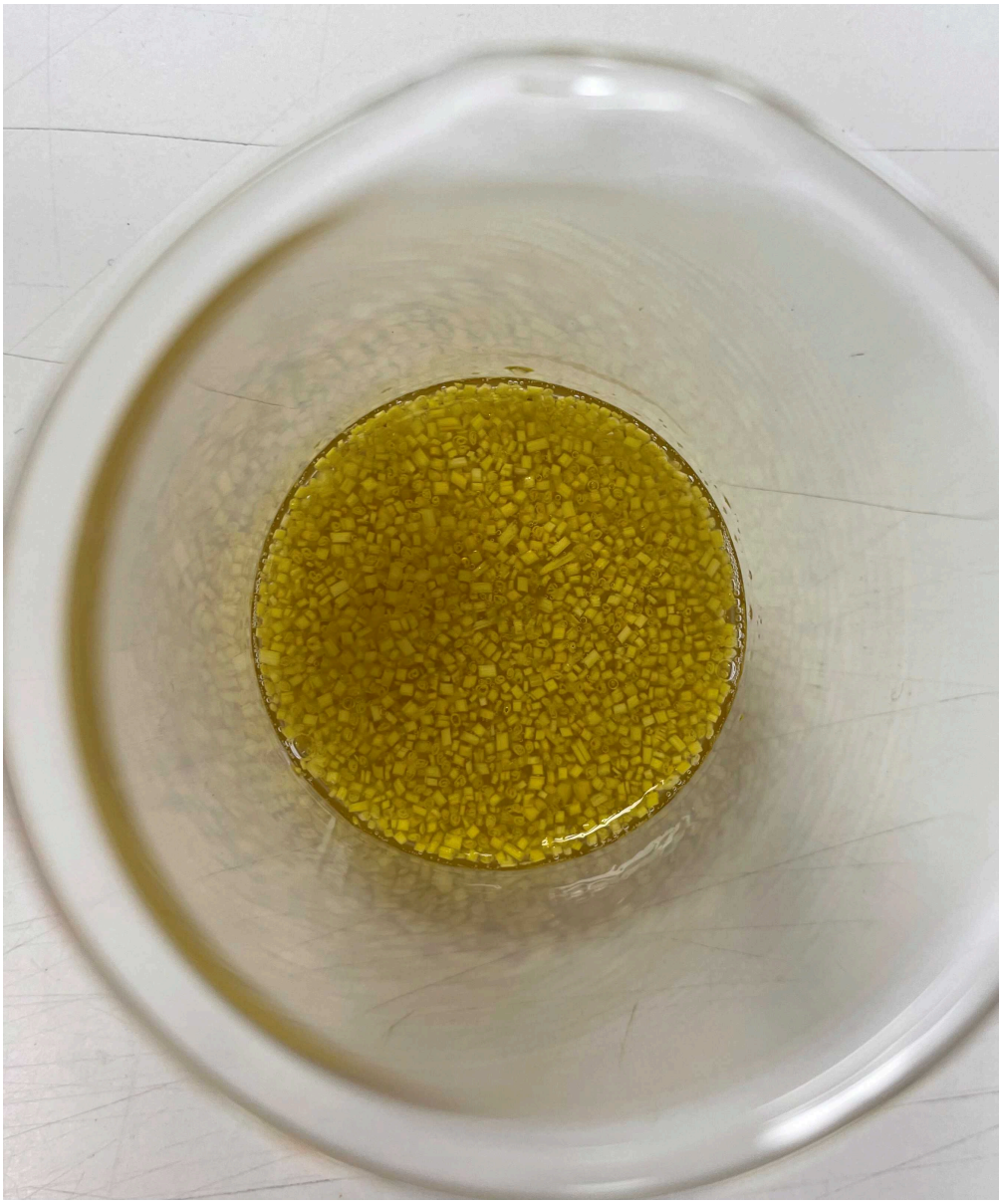
Bunched up leaves ready to be cut.



Cut leaves need to remain in buffer and not dry out.

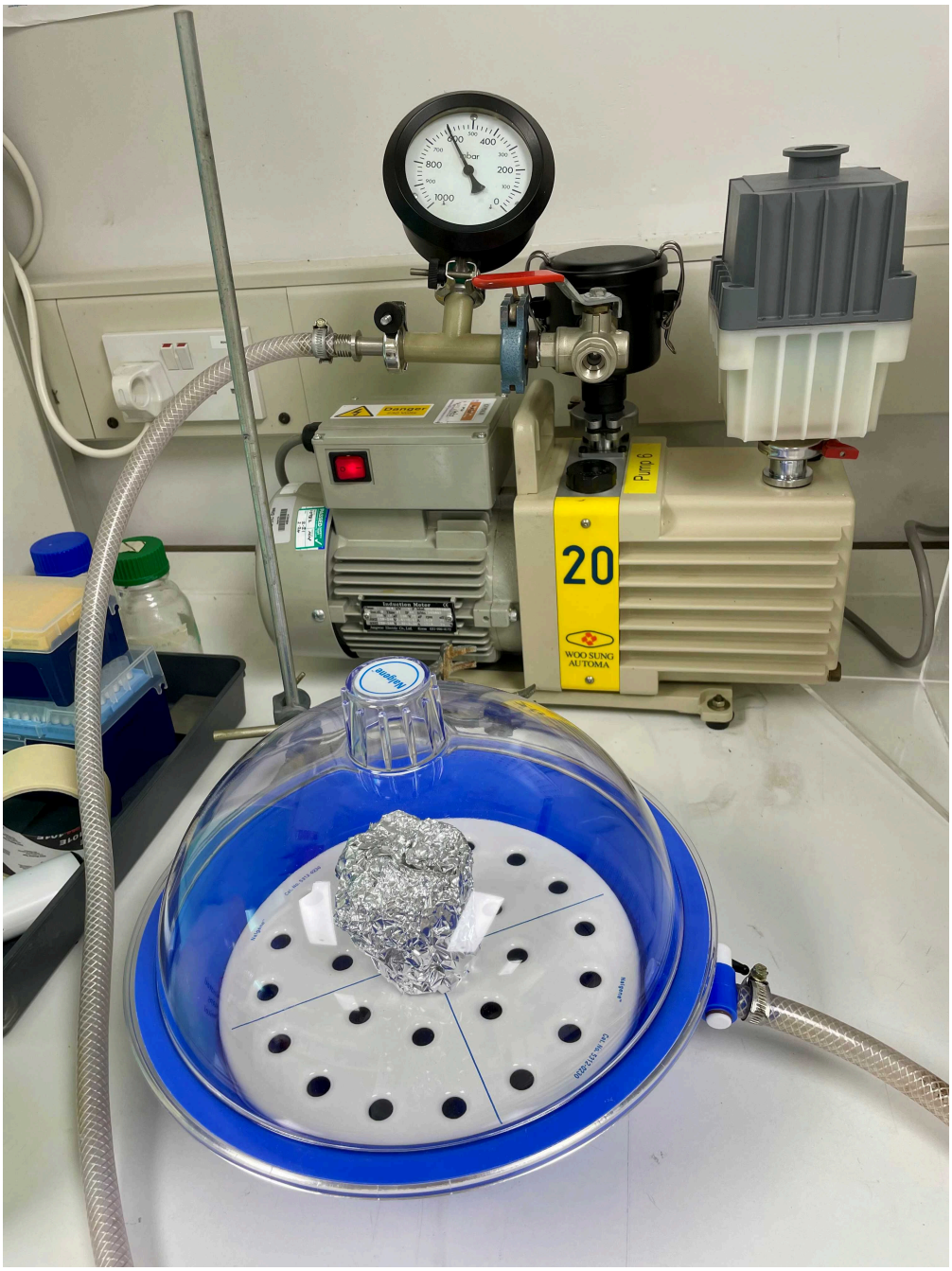
10 Add some plasmolysis buffer (enough to cover the cut leaves) and keep for 10 min in the dark (no shaking).

11 Remove excess plasmolysis buffer and transfer cut leaves (using the razor blade) into a 100 ml glass sterile beaker and add all 10 ml of the enzyme solution. Gently swirl to mix. Ensure that all leaves are covered by the enzyme solution.



Leaf segments in enzyme solution.

12 Place the beaker in a desiccator (wrap the beaker in foil to keep the cells in the dark but make a small gap at the top to allow the air to flow and the vacuum to work). Turn on the pump (go to 600 mbar) and vacuum infiltrate for 30 minutes.

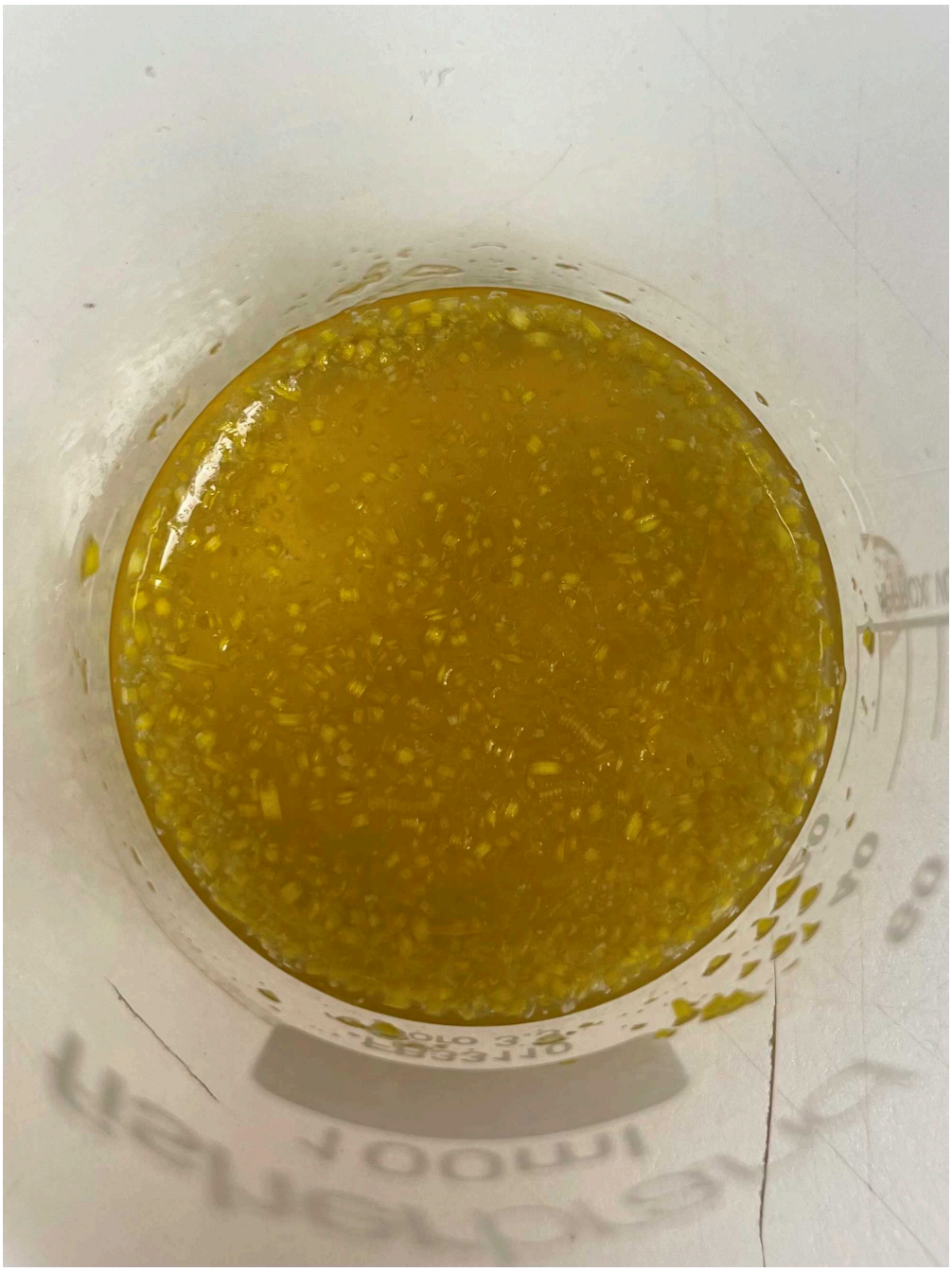


Vacuum infiltration of the enzyme solution in the leaf segments via pump at 600 mbar for 30 min.

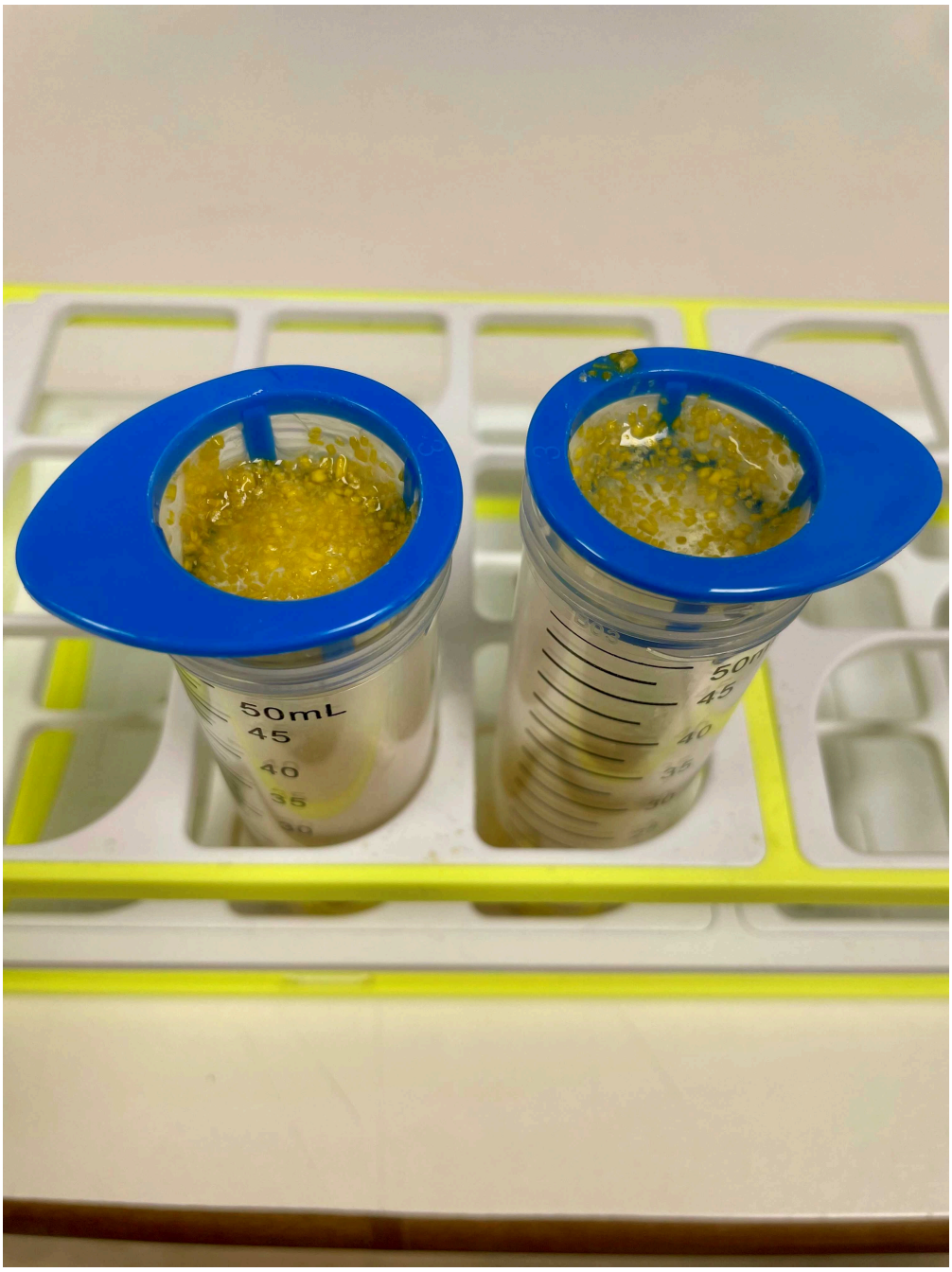
13 Remove the beaker from the desiccator. Close the gap with extra foil to ensure the cut leaves are in complete darkness. Then incubate in the dark for 4 hours (25°C shaking at 40 rpm) to allow the enzymes to degrade the cell wall of the cells in suspension.

14 Put W5 on ice.

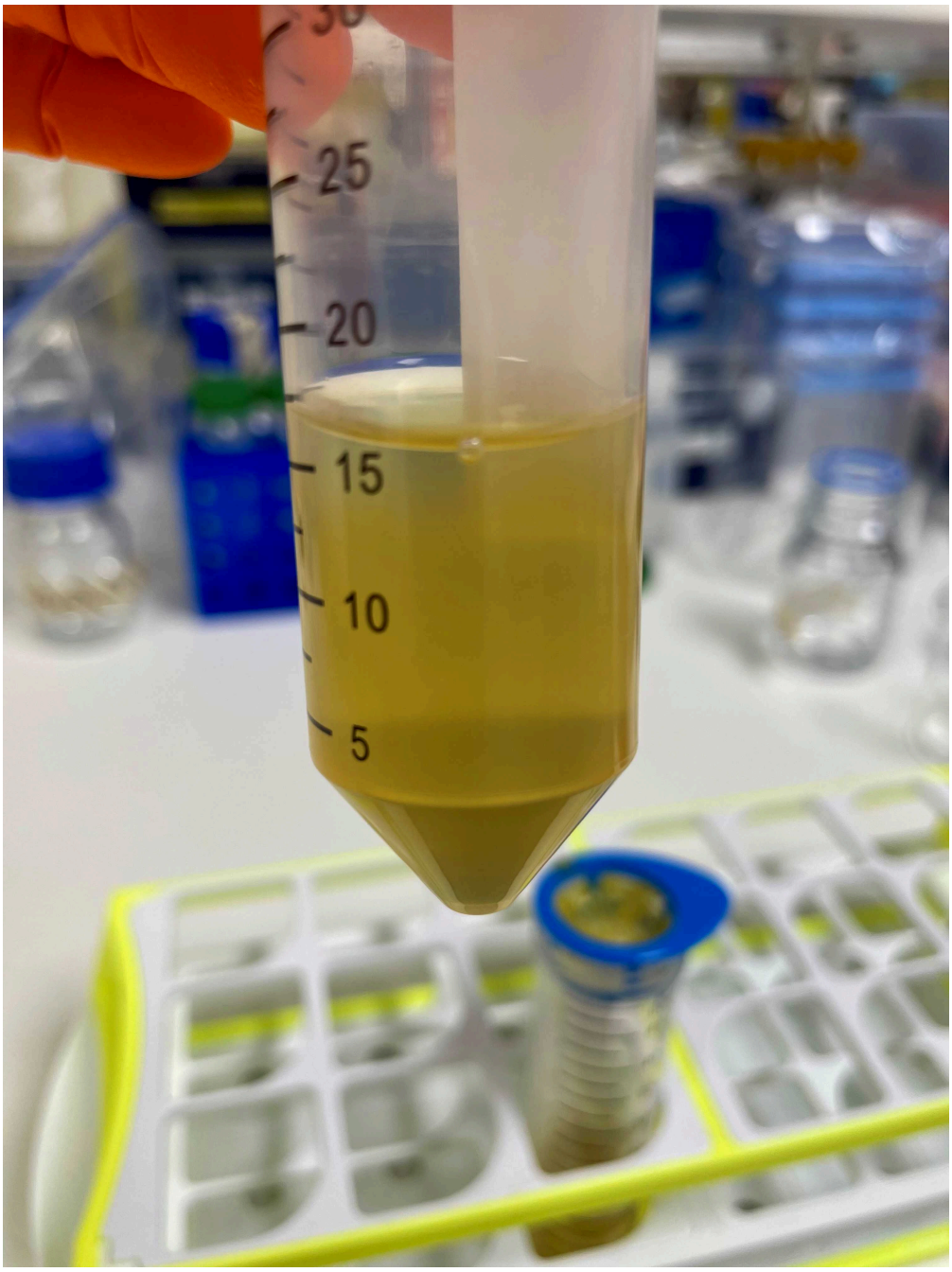
15 Wet the nylon mesh of two 40 µm filters by pipetting 1 ml of plasmolysis buffer in each. Place the filters in two 50 ml Falcon tubes. Swirl the digested cells in the enzyme solution round and gently pour through both filters (keep the Falcon tube + filter at a 45° angle when tipping the liquid into the filter to limit possible cell rupture, put half in each tube – don't swirl the solution while it filters, just let it go through gently to limit the amount of debris going through).



Digested cells from leaf segments after 4 hours of enzyme solution incubation.



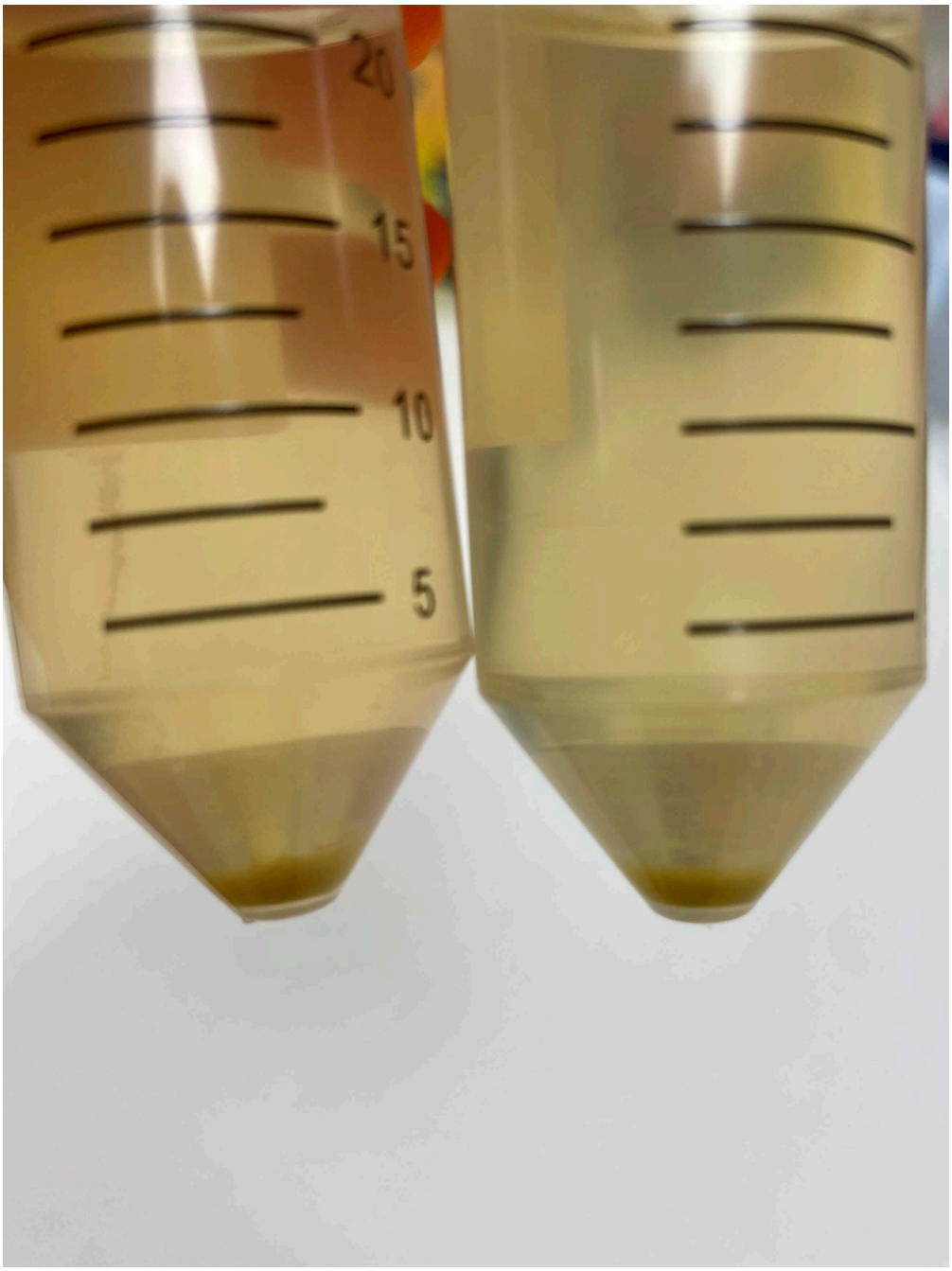
16 Add a few ml of plasmolysis buffer back into the glass beaker and swirl, pour through mesh into the same tube and repeat this process until all digested cells in enzyme solution have been collected. Typically end up with about 20 ml to 30 ml of filtered cells in solution per tube.



Filtered solution.

Filtered solution in 2 tubes ready for step 17.

17 Centrifuge for 3 mins at 80 g (slow brake and acceleration) at 4°C and discard the supernatant without disturbing the cell pellet.

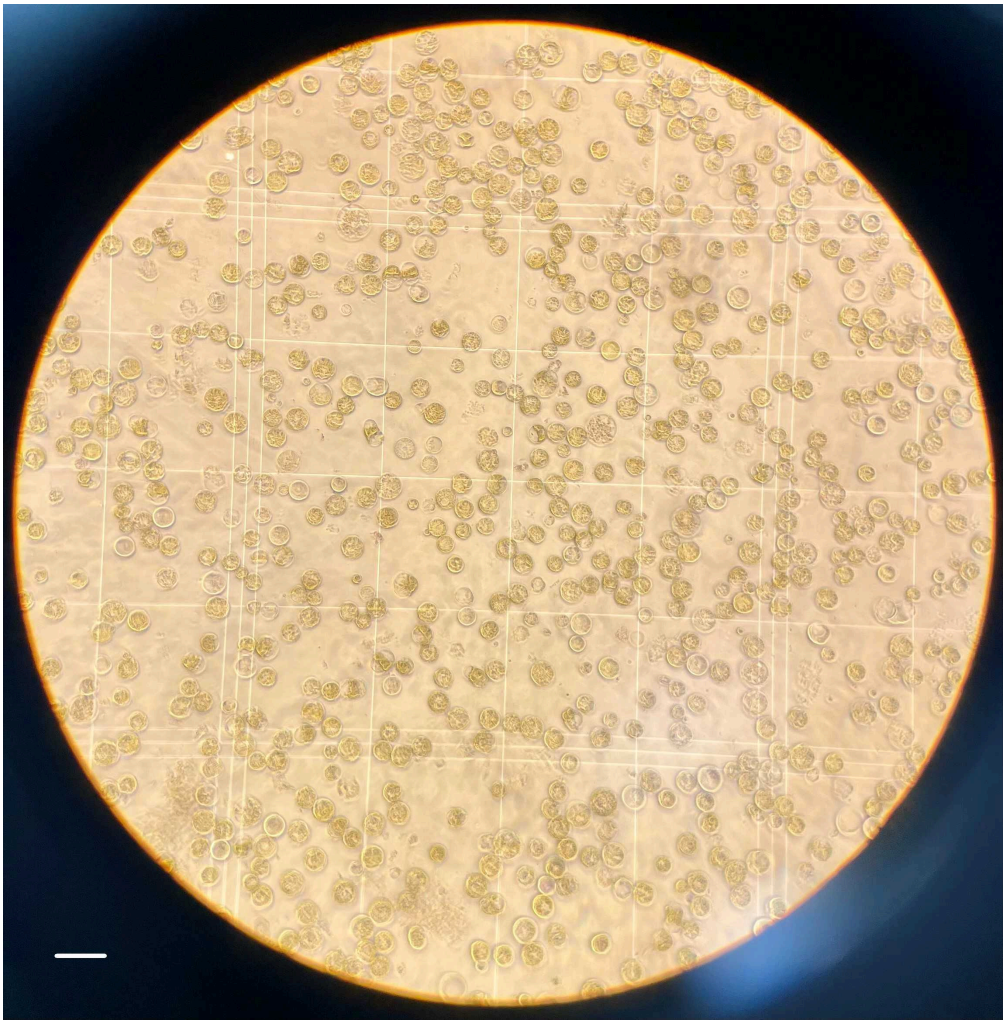


Pelleted protoplasts after filtering and centrifugation.

18 Resuspend in 3 ml of ice cold W5 solution. Resuspension should be very gentle: just move the tube from vertical to a 45° angle whilst simultaneously spinning the tube round (on the longitudinal axis). It might take a few minutes to get the protoplasts fully resuspended but it is important to be very gentle during resuspension steps to ensure the cells don't burst.
NB: Always resuspend in this way in following steps.

Place the tubes on ice for 30 mins which will allow the protoplasts to gradually sink into a pellet at the base of the tube.

19 Meanwhile, count protoplasts with a hemacytometer (10x magnification).
NB: If there is a lot of debris, filter the protoplast solution once more (steps 15 to 18), as debris will interfere with transformation success and with cell sorting.

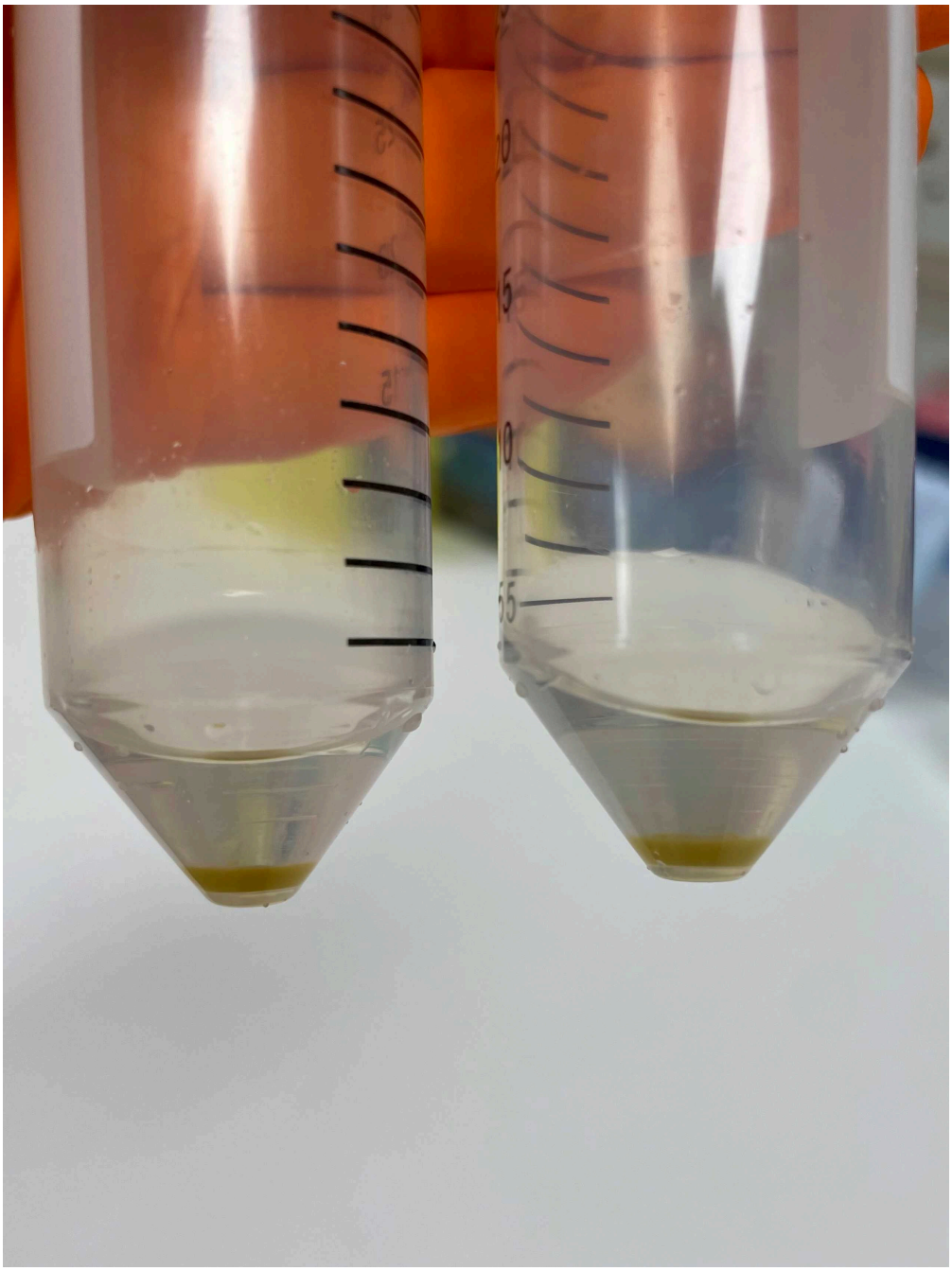


Protoplasts on a hemacytometer slide visualised at 10x magnification. Scale bar = 100 µm.

20 Start to make the PEG solution. Dissolve and vortex 15 mins before use. It may require 10 to 15 mins of vortexing but it needs to be as fresh as possible - DO NOT HEAT.

Protoplast transformation

21 Centrifuge the protoplasts for 3 min at 80 g (slow brake and acceleration). Remove and discard the supernatant without disturbing the cell pellet.



Protoplast pellets after ice incubation and centrifugation.

22 Resuspend the protoplasts to ~ 1 × 10^6 cell per ml in MaMg (typically this will be around 2 to 4 ml). Use this step to combine the contents of the 2 tubes back into one.

23 For each transformation, do the following 3 steps together (per transformation) and **without delay in between steps** (this is very important).
NB: Calculate how many transformations are needed according to the number of reps and treatments required per experiment (see steps 34 and 36). It is recommended to carry out several independent transformations and then pool them together the next day and aliquot the pooled samples as technical reps per treatment.
Example table:

	TREATMENT			
	N	DEX	CHX	DEX+CHX
Rep 1	1.2 ml	1.2 ml	1.2 ml	1.2 ml
Rep 2	1.2 ml	1.2 ml	1.2 ml	1.2 ml
Rep 3	1.2 ml	1.2 ml	1.2 ml	1.2 ml

Example table for 1 construct.

In this example, 12 × 1.2 ml = 14.4 ml of transformed protoplasts in PIM will be needed. Therefore, 10 transformations will need to be carried out (14.4 ml / 1.5 ml = 9.6 transformations).

24 Pipet 10 µl of plasmid (at 1-2 µg/µl) into a 2 ml Eppendorf tube.

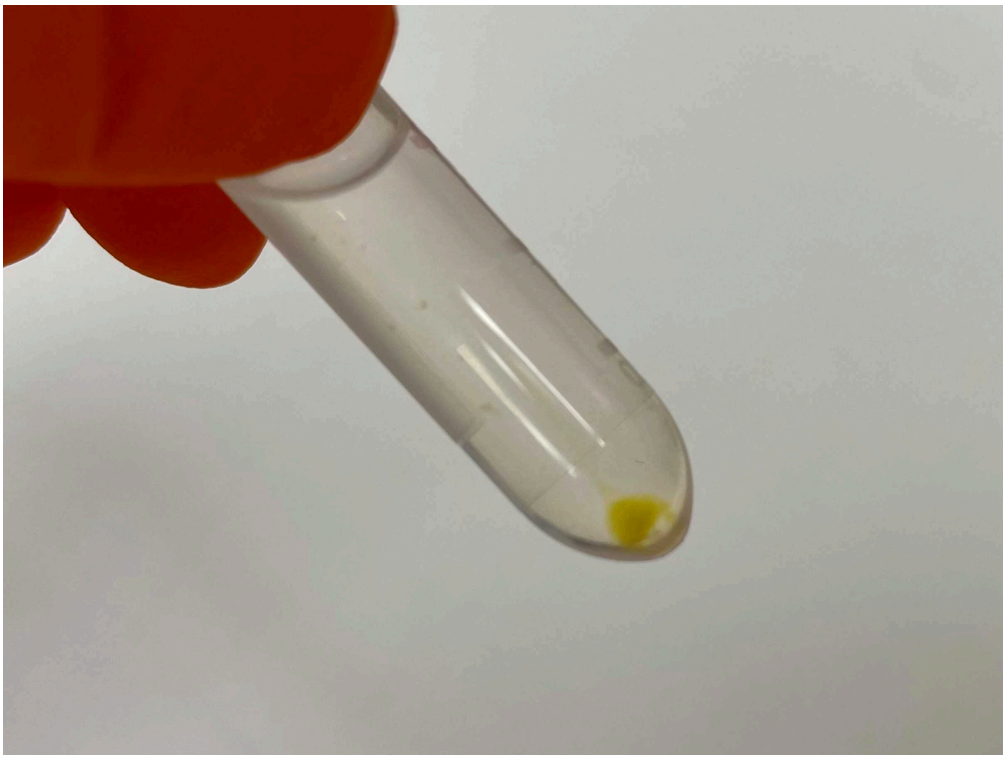
25 Immediately add 100 µl of protoplasts to the DNA, mix very gently but well by moving the tube nearly horizontal and tipping it sideways slowly.

26 Immediately add 110 µl of PEG. Mix very gently but well by moving the tube nearly horizontal and tipping it sideways slowly.

27 Incubate at 23°C for 15 mins in the dark, ideally at a 45° angle. **Do not exceed 15 min** as this will lower the transformation efficiency.

28 Add 1.5 ml of W5 solution to stop the transformation and mix gently.

29 Centrifuge at 80 g (slow brake and acceleration) for 90 seconds then remove the supernatant without disturbing the cell pellet.



Transformed protoplasts after centrifugation.

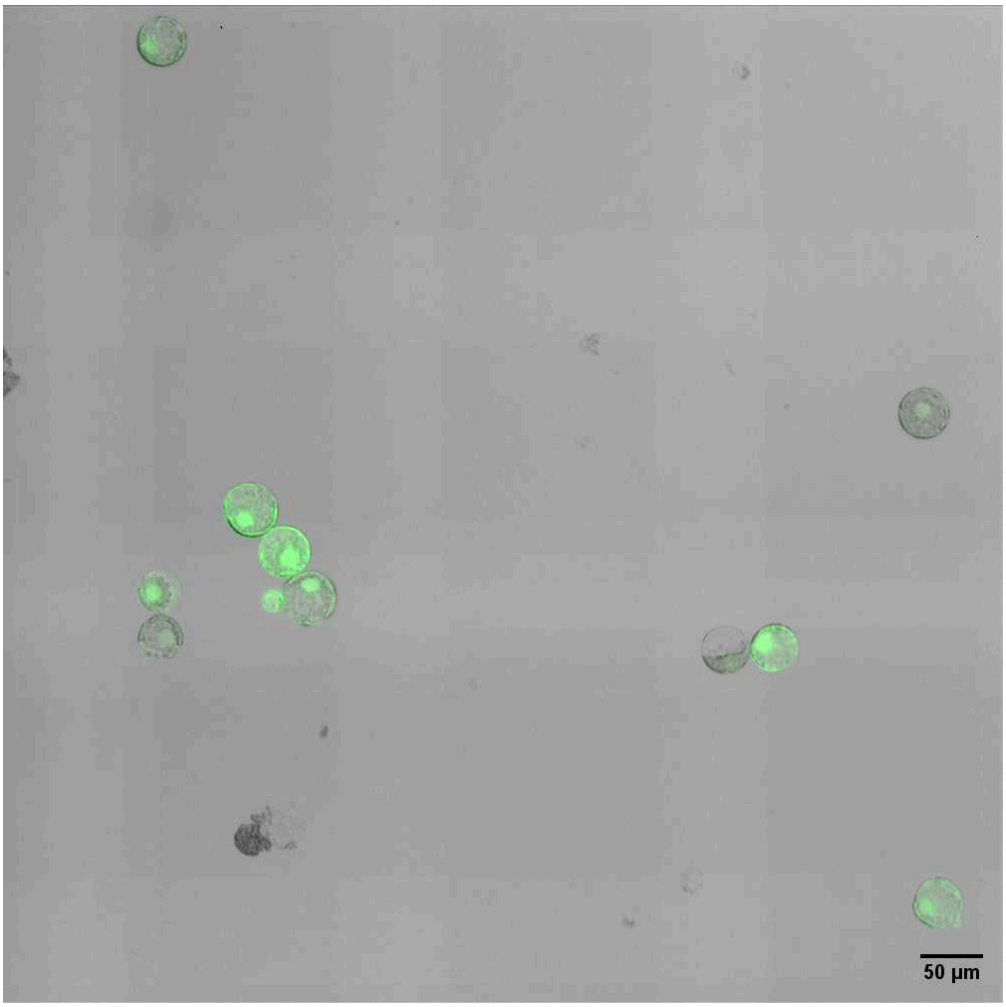
30 Resuspend the cells gently in 1.5 ml of PIM.

31 Incubate the transformed protoplasts in the dark at 23°C overnight (usually room temperature) ideally at a 45° angle (no shaking).

Protoplast treatment and FACS

32 Resuspend the cells very gently the next day (as they will have settled down to a pellet overnight).

33 Check the cells on an epifluorescence microscope with settings for bright field and for GFP/RFP. Transformation efficiency will typically yield 50-75 % of transformed protoplasts.



Transformed protoplasts (expressing GFP) visualised on a Zeiss Axio Observer Z1 microscope with a 10x objective.

34 For all successful transformations with the same construct, transformed protoplasts in PIM are then pooled together into one tube (this reduces variability between individual transformations). They are then split in equal volumes (ideally 1.2 ml each) into 4 tubes (2 ml Eppendorf tubes) for each treatment: N (non treated - control), DEX (treated with dexamethasone alone), CHX (treated with cycloheximide alone) and DEX+CHX (treated with dexamethasone and cycloheximide).
DEX and CHX working solutions are prepared as follows from the stock solutions:
- 10 µl of DEX stock (at 10 mM) plus 90 µl of PIM: makes a 1 mM working solution.
- 1 µl of CHX stock (at 100 mg/ml = 355 mM) plus 99 µl of PIM: makes a 3.55 mM working solution.
NB: adjust volumes according to number of samples for each treatment.
Treatments are administered by adding 35.5 µM CHX (12 µl of working solution into 1.2 ml of protoplast/PIM solution) for 20 minutes (before FACS or before DEX treatment); and/or by adding 10 µM DEX (12 µl working solution into 1.2 ml of protoplast/PIM solution) for 3

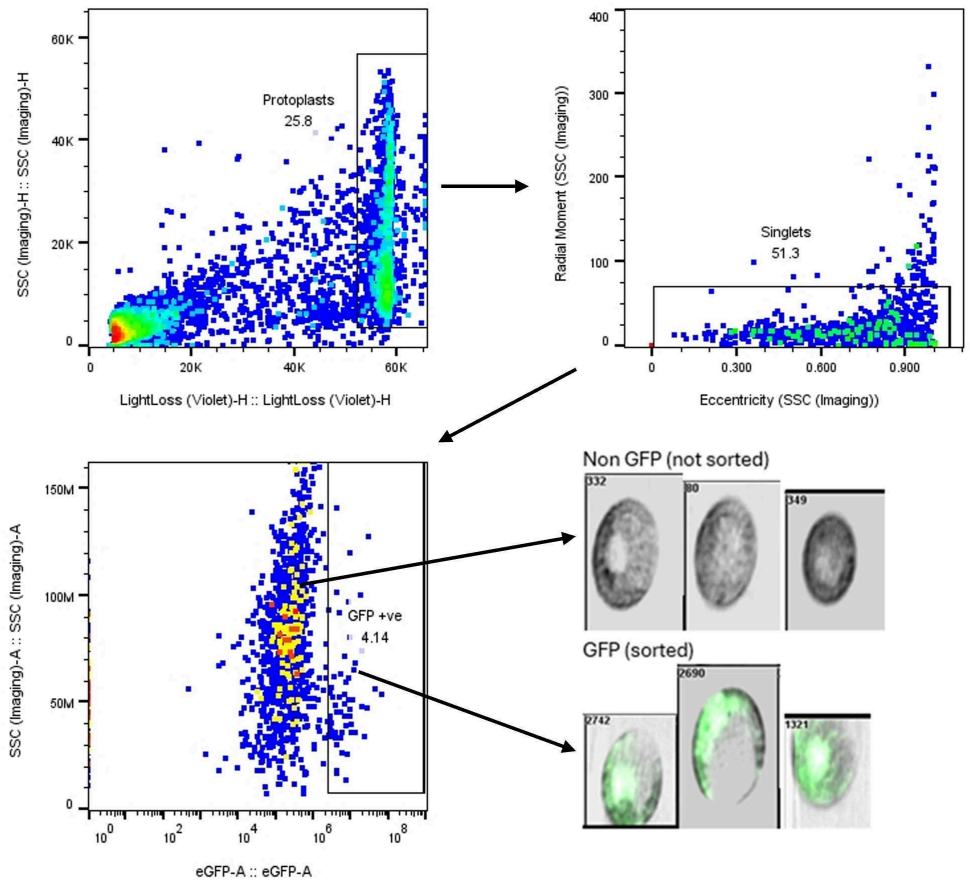
hours (before FACS). Treated cells are incubated at room temperature in the dark (no shaking).
NB: It is possible to omit the DEX alone sample as described in Note 13 of Brooks *et al.*, 2023[2].

35 Treatment times are staggered (20 to 30 minutes apart) in order to be ready for FACS at different times, allowing for enough sorting time per sample (typically 20 to 30 minutes).

36 Each treatment is repeated 3 times (3 technical reps), therefore enough protoplasts need to have been transformed to allow for enough reps and treatments.

37 Treated samples are sorted via FACS (Fluorescence Activated Cell Sorting) using a 5 laser BD FACS Discover S8 (Sort mode: Yield, Nozzle size: 130 µm, Drop frequency: 15 kHz, Pressure: 6.60 PSI) at room temperature. Spectral unmixing is performed using BD FACS Chorus software (Version 5.4.0). Protoplasts are gated using Side scatter (height) imaging vs Light loss (height) Violet to isolate larger particles, Radial moment (Side scatter) imaging vs Eccentricity (Side scatter) imaging to exclude clumps and doublets, and eGFP (area) vs Side scatter (area) Imaging to distinguish GFP positive protoplasts. Sorted protoplasts are collected in 5 mL FACS tubes containing 350 µl of buffer RLT (from the QIAGEN RNeasy microkit). The 350 µl buffer + sorted protoplasts were then transferred to a 2 ml RNase-free Eppendorf tube placed on dry ice until transferred to a -70°C freezer. Samples are kept at -70°C until RNA extraction.
NB: Ideally a minimum of 3500 transformed cells (GFP or RFP) need to be collected per sample to allow for enough RNA to be extracted.

38



RNA extraction and RNAseq analysis

39 RNA is extracted from each sample using the QIAGEN RNeasy microkit. RNA is eluted in 14 µl of RNAse-free H_2O. Typically, 10 to 40 ng/µl of RNA are recovered.

40 RNA samples are then sent for sequencing (mRNA library preparation - polyA enrichment; 150 bp paired-end reads; 30 to 40 M reads).

41 Differential gene expression analysis is carried out to identify target genes. Our standard pipeline involves read trimming with Trimmomatic, mapping reads to the wheat transcriptome using Kallisto and DESeq2 for differential expression analysis.

42 As described in Brooks *et al.*, 2019[3], the TF-GR complex binds to a heat shock protein (HSP) present in the cytoplasm of the cells. When DEX treatment is applied, this interaction is disrupted, which leads to the TF-GR complex being enabled to move to the

nucleus and the TF is then able to interact with its targets. Pre-treatment with CHX, a translation inhibitor, blocks downstream regulation of secondary targets of the TF. Therefore, DEX treatment alone identifies direct and indirect targets of the TF. DEX treatment combined with a CHX pre-treatment identifies direct targets only.
So in terms of DEG analysis:
- N (non-treated) versus CHX comparison identifies differentially expressed genes as a result of the CHX treatment.
- N versus DEX comparison identifies all targets (direct and indirect) of the TF of interest.
- CHX versus CHX+DEX comparison identifies direct targets of the TF of interest.

Protocol references

1. Bargmann BO, Marshall-Colon A, Efroni I, Ruffel S, Birnbaum KD, Coruzzi GM, Krouk G. TARGET: a transient transformation system for genome-wide transcription factor target discovery. Mol Plant. 2013 May;6(3):978-80. doi: 10.1093/mp/sst010. Epub 2013 Jan 18. PMID: 23335732; PMCID: PMC3660954.

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