Modified Single-Cell Transient Gene Expression Assay in Barley Epidermal Cells

Shiwei Bai, Cheng Chang, Xinyun Han, Qian-Hua Shen*

State Key Laboratory of Plant Cell and Chromosome Engineering, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China

*For correspondence: qhshen@genetics.ac.cn

[Abstract] Transient gene expression via biolistic particle delivery is a widely used technique for gene functional analysis in plants. In this protocol we describe a modified single-cell transient expression assay through transformation with a particle inflow gun of the model PDS-1000/He system (Bio-Rad). This assay was originally optimized for analyzing cell death activity and disease resistance function of the barley MLA (mildew locus A) disease resistance proteins against the powdery mildew fungus, which can be further adopted for other purposes for other types of plant proteins and in some other plant species, including *Arabidopsis thaliana*.

Materials and Reagents

1. Barley (*Hordeum vulgare* L.) plants, 1 week old seedlings
2. Powdery mildew strain(s), fresh conidiospores as inoculum
3. Benzimidazol (Genview, catalog number: 51-17-2)
4. Agar (Japan, plant cell culture tested)
5. $CaCl_2$ (Sigma-Aldrich, catalog number: C7902)
6. X-gluc (Inalco, catalog number: 1758-0600)
7. Coomassie Brilliant Blue R-250 (Amresco, catalog number: 0472)
8. Ethanol (Beijing Chemical Works)
9. Glycerol (Beijing Chemical Works)
10. Methanol (Beijing Chemical Works)
11. Lactic acid (Beijing Chemical Works)
12. Plasmid
13. Reporter DNA
14. $K_3Fe [CN_6]$ (Sinopharm Chemical Reagent, catalog number: 10016718)
15. Triton X-100 (AMRESCO, catalog number: 0694)
16. Spermidine (Sigma-Aldrich, catalog number: S-4139) (see Recipes)
17. GUS staining solution (See Recipes)
18. Destaining solution (See Recipes)
19. Benzimidazol plates (see Recipes)

20. Coomassie blue solution (see Recipes)

Equipment

1. PDS-1000/He delivery system (Bio-Rad Laboratories)
2. Macrocarrier (Bio-Rad Laboratories, catalog number: 1652335)
3. Rupture disc: 900 psi (Bio-Rad Laboratories, catalog number: 1652328)
4. Centrifuges (Eppendorf, catalog number: 5424)
5. Fluorescence microscope (Carl Zeiss, Axio Scope. A1)
6. pH meter (Mettler Toledo, FE20K)
7. Gold microcarrier: 1.0 μm in diameter (Bio-Rad Laboratories, catalog number: 165-2263)

Procedure

A. Preparations
1. Inoculate plants with powdery mildew spores to prepare inoculum and sow barley seeds to grow plants for bombardment one week in advance. The plants were grown in a growth chamber under a 16 h/8 h, 20 °C/18 °C day/night cycle with 70% relative humidity.
2. Prepare Benzimidazol plates one day in advance.

B. Bombardment
1. Cut primary leaves and put them on Benzimidazol plates with adaxial side up (Figure 1), 3-5 leaves per petridish (90 mm) per shot, incubate at least 4 h before shooting.

Figure 1. Picture showing preparation of Benzimidazol agar plate with barley leaves for bombardment. 5-6 barley primary leaves were detached from 1 week old barley seedlings and put side by side with adaxial side up on prepared Benzimidazol agar plate.

2. Prepare gold particles (20 shots):
   a. Weigh 9 mg gold particles in a 1.5 ml tube.
   b. Add 1 ml 70% ethanol, vortex 5 min, sediment particles for 15 min on bench.
   c. Spin 2 sec. (about 2,000 rpm), discard supernatant.
   d. Repeat 3 times: add 1 ml sterile H_2O, vortex 2 min, sediment 1 min, spin 2 sec. (about 2,000 rpm), discard supernatant.
   e. Add 1 ml of 50% glycerol (in water), vortex (gold particles can be stored at -20°C for 2-3 weeks).
3. Coat the gold particles (use 50 µl gold particle solution for one shot):
   a. Vortex gold particle for at least 5 min.
   b. Mix equal molar plasmid and reporter DNA (*e.g.* GUS or GFP reporter), do not use more than 2 µg DNA in total, add ddH_2O when volume is less than 5 µl.
   c. Aliquot 50 µl gold particles into each empty tube, then add DNA solution.
   d. While vortexing, add: 50 µl 2.5 M $CaCl_2$ drop-by-drop, then 20 µl 0.1 M spermidine, vortex for 3 min in total.
   e. Sediment particles for 1 min, spin 2 sec. (2,000 rpm), discard supernatant.
   f. Add 140 µl 70% ethanol, vortex, spin 2 sec. (2,000 rpm), discard supernatant.
   g. Add140 µl 100% ethanol, vortex, spin 2 sec. (2,000 rpm), discard supernatant.
   h. Add 15 µl 100% ethanol, vortex, store on ice until used.
4. Bombard, for each shot repeat the following steps:
   a. Fix the macrocarriers in macrocarier holder, suspend particles by pipetting, and apply the particles onto the macrocarrier. Dry on the bench.
   b. Dip rupture disc (900 psi) in 100% (v/v) 2-propanol and subsequentlyplace it into rupture disk retaining cap, add few more drops of 2-propanol.
   c. Insert macrocarrier holder with stop-screen in stop screen holder at position 1 (from top) (Figure 2).
   d. Insert petridish with leaves at position 3 (Figure 2).
   e. Apply vacuum up to 27 inches of mercury, trigger the shot.
   f. Arrange leaves on the petridish, put in incubator.
   Note: We put the leaves on the dish side by side with adaxial side up (Figure 1).

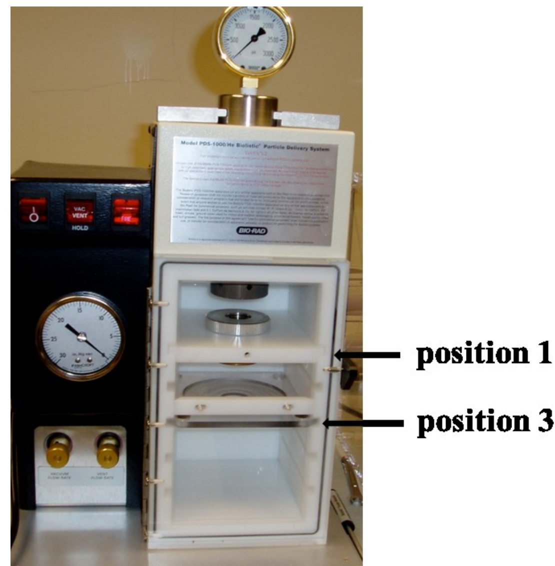

Figure 2. Picture showing the PDS-1000/He delivery system. Indicated are position 1 and 3 that reserved for macrocarrier holder and patridish holder, respectively.

5. (Omit this step if fungal inoculation is not necessary)
   Inoculate with powdery mildew condiospores at least 4 h after bombardment.

C. For GFP index scoring
   36-48 h after bombardment count GFP expressing cell numbers using fluorescence microscope.
   Note: The total number of cells here is the sum of compatible (haustorium, seccondary hyphae) and incompatible (only appressorium) on GUS expressing cells. We score all of the five leaves and at least 60 cells were scored.

D. For fungal Haustorium index scoring
   1. 48 h after fungal spores inoculation
      Stain leaves for GUS expression: put leaves into 15 ml falcon tube containing about 8 ml X-gluc staining solution, vacuum infiltrate 5 min for 3 times, and incubate overnight to 24 h at 37 °C.
   2. 1 day after GUS staining
      Remove GUS staining solution, add about 10 ml destaining solution, store at RT at least 2 days.
   3. When time available:
      Stain for the fungus:
      a. Transfer leaves to large volume of ddH$_2$O for 1 h.

   b. Stain in Coomassie solution for few seconds.
   c. Wash twice in water.
   d. Mount on microscope slide in 50% glycerol. Once on the slide the samples should be scored within few days.
4. Score compatible (visible intracellular haustorium, and sometimes secondary hyphae on leaf surface) and incompatible (only fungal appressorium) interaction cell/site for GUS expressing cells.

Recipes

1. Benzimidazol plates
   1% agar in water with 85 µM Benzimidazol (from 8.5 mM stock solution in water, 100x)
   Note: The pH value for these plates is about 6.5. It is not necessary to adjust the pH value.
2. Spermidine
   0.1 M solution, 1 g solution mix with 67.8 ddH_2O, filter sterilized.
   Aliquot and stored at -20 °C (note it's very hygroscopic and air sensitive, close and put back to freezer immediately when done)
   2.5 M $CaCl_2$ in water, sterile filtrate store in RT
3. GUS staining solution
   0.1 M Na_2HPO_4/NaH_2PO_4 (pH 7.0)
   10 mM Na-EDTA
   5 mM $K_4Fe\ [CN_6]$
   5 mM $K_3Fe\ [CN_6]$
   0.1% Triton X-100 (v/v)
   20% methanol (v/v)
   1 g/L X-gluc
   Adjust to pH 7.0
4. Destaining solution: Stock solution
   50% glycerol
   25% lactic acid
   25% H_2O
   Dissolve 1 volume stock solution in 2 volumes ethanol.
5. Coomassie blue solution
   0.6% coomassie blue (w/v) in 100% (v/v) methanol/or ethanol

Acknowledgments

This protocol is adapted from Shirasu *et al*. (1999); Shen *et al*. (2012) and Bai *et al*. (2012).

References

1. Bai, S., Liu, J., Chang, C., Zhang, L., Maekawa, T., Wang, Q., Xiao, W., Liu, Y., Chai, J., Takken, F. L., Schulze-Lefert, P. and Shen, Q. H. (2012). Structure-function analysis of barley NLR immune receptor MLA10 reveals its cell compartment specific activity in cell death and disease resistance. *PLoS Pathog* 8(6): e1002752.
2. Shen, Q. H., Zhou, F., Bieri, S., Haizel, T., Shirasu, K. and Schulze-Lefert, P. (2003). Recognition specificity and RAR1/SGT1 dependence in barley Mla disease resistance genes to the powdery mildew fungus. *Plant Cell* 15(3): 732-744.
3. Shirasu, K., Nielsen, K., Piffanelli, P., Oliver, R., and Schulze-Lefert, P. (1999). Cell-autonomous complementation of *mlo* resistance using a biolistic transient expression system. *Plant J* 17: 293-299.