Bimolecular Fluorescence Complementation (BIFC) Protocol for Rice Protoplast Transformation

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[Abstract] After the plant cells are removed the cell walls by digestive enzyme, the plant protoplasts still have good cell activity. The protoplasts can be used to transiently express proteins of target genes in living plant cells through polyethylene glycol (PEG) mediated transformation. The purpose of this method is to employ the rice protoplasts and Green fluorescent protein (GFP) as an experimental system to observe the protein interactions *in vivo*. Meanwhile a 505~530 nm emission filter is used in confocal microscope to eliminate the interference of the autofluorescence from plant cells. The phenomenon of plant cell body spontaneous fluorescence can be eliminated by confocal observation.

Materials and Reagents

1. Rice (*Oryza sativa* L.) grain seeds
2. Mannitol (Sigma-Aldrich)
3. Morpholinoethane sulfonic acid (MES) (Sigma-Aldrich)
4. Cellulose R-10 (Yakult Honsha)
5. Macerozyme R-10 (Yakult Honsha)
6. Bovine serum albumin (BSA) (Sigma-Aldrich)
7. Carbenicillin/Ampicillin (Amresco)
8. β-Mercaptoethanol (β-ME) (Amresco)
9. Polyethtlene glycol (PEG) 4000 (Sigma-Aldrich)
10. Enzyme solution (see Recipes)
11. PEG4000 solution (see Recipes)
12. W5 solution (see Recipes)
13. MMG solution (see Recipes)

Equipment

1. Shaker P270 (Chinese Academy of Sciences, Wuhan Scientific Instrument Plant)

2. Collagen-coated 35-mm-diameter glass-base dish
3. Vortex XW-80A (JiaPeng Techno)
4. Nylon mesh (35 μm) (EMD Millipore)
5. Vacuum pump
6. 50 ml with round bottom centrifuge tube
7. 2 ml centrifuge tube
8. Tabletop centrifuges (Eppendorf 5810R and 5417R)
9. Collagen-coated 35-mm-diameter glass-base dish (Asahi Techno Glass Corporation)
10. Confocal microscopy (Olympus, model: FV1000)
11. OLYMPUS FV1000 system (Fluoview Ver.1.7b Viewer) (Olympus)

Procedure

A. Protoplast Preparation

1. Germinate 100 of the sterilizing rice grain seeds on wet gauze under darkness at 28 °C for about one week.
2. When the etiolated seedlings grow to about 7~8 cm, collect the etiolated seedlings, and immediately use a sharp blade to cut the seedlings into ~0.5 mm segments, then, have the segments fully immersed in 50 ml 0.6 M mannitol solution for 10 min.
3. Transfer the seedling fragments into Enzyme Solution.
4. Using vacuum pump to remove air in the tissues to help them being completely precipitated in the Enzyme Solution.
5. Keep the tissue-immersed solution in darkness at 28 °C, and agitated at 80 rpm on a shaker for ~4 hours.
6. Wash the nylon net (35 μm) with ddH_2O and then wet it with W5 solution for 3-5 min before filtering the protoplast, and then the enzyme digested samples are filtered to a 50 ml centrifuge tube with round bottom. Slightly twist the nylon net to improve the yield.
7. Centrifuge the filtration at 100 *x g* for 5 min, discard the supernatant and remove the residual liquid as much as possible with pipette, then add 10 ml pre-cooled W5 solution to resuspend the protoplast pellet by gentle swirling.
8. Incubate the tube for 30 minutes on ice. (The following operations are under room temperature.)
9. Precipitate the protoplast by centrifugation (100 *x g* for 5 min). Discard the supernatant and remove the residual liquid, then gently add 1 ml MMG solution to resuspend the protoplasts. Finally, adjust the protoplast density to 2 x 10^5 cells/ml under microscope (40x).

B. Protoplast Transformation

Note: Before protoplast transformation, please prepare the BIFC expression vectors according to the protocols of Walter et al. (2004), and we recommend to refer the information on how to prepare plasmid DNA using the economical CsCl gradient on the website of Sheen lab (http://genetics.mgh.harvard.edu/sheenweb/protocols.html).

1. Aliquot 100 µl of the protoplasts to 2 ml centrifuge tubes.
2. For transformation, empty vectors pUC-SPYNE/pUC-SPYCE and bZIP6-YFP^N/bZIP6-YFP^C are used as negtive and positive controls, respectively. 20 µl of the BIFC vectors (≥ 1-2 µg/µl, 10 µl per vector), negative control and positive control are added to each tube, respectively, and then mix gently.
3. Add equal volume (120 µl) PEG solution to each tube and mix well.
4. Incubate the mixture for 15 min for transformation.
5. Add 480 µl of W5 solution to stop the transformation.
6. Centrifuge the solution at 100 *x g* for 2 min, and discard the supernatant.
7. Add 1 ml W5 solution to gently resuspend the protoplast pellet, and add 1 µl Carbenicillin (50 mg/ml) before transferring the protoplasts to culture plate, culture at room temperature for 16-20 h in darkness to allow expression of the BIFC proteins.
8. Before confocal observation, the transformed protoplasts should be centrifuged at 100 *x g* for 2 min and remove most of the supernatant, then resuspend the protoplasts.

C. Confocal observation

1. Transfer the protoplast into a collagen-coated 35-mm-diameter glass-base dish for microscopy observation.
2. Collection of the confocal fluorescence signals was performed on Olympus FV1000 system.
3. The interference from autofluorescence problem in experiment can be eliminated by optical sectioning generated in confocal microscopy. We choose using excitation with the 488-nm line of an argon laser and a 505~530 nm band-pass emission filter.
4. Under this observation regime, the positive control show strong yellow fluorescence, and the negative control is black. This confirms all of the operations above are reliable for the BIFC observation.

Recipes

1. Enzyme solution (10 ml)

Mannitol (0.6 M)	1.093 g
MES (10 mM, pH 5.7)	1 ml (100 mM stock solution)

Cellulose R-10 (1.5%) 0.15 g
Macerozyme R-10 (0.75%) 0.075 g
BSA (0.1%) 0.01 g
$CaCl_2$ (1 mM) 0.1 ml (100 mM stock solution)
Carbenicilli (0.25 g/ml) 2 µl
β-ME 4 µl
Add ddH_2O to 10 ml
55 °C 10 min
Natural cooling (Preparing it when you use)

2. PEG4000 solution

Mannitol (0.6 M) 1.093 g
$CaCl_2$ (100 mM) 0.111 g
PEG4000 (40%) 4 g
Add ddH_2O to 10 ml
Using 1 M KOH to adjust the pH to 7.5~8.0
Aliquot with 1.5 ml centrifuge tube and preserve at -20 °C

3. W5 solution

W5 (100 ml)		
154 mM NaCl	NaCl	0.9 g
125 mM $CaCl_2$	$CaCl_2$	1.39 g
5 mM KCl	KCl	5 ml 100 mM stock solution
5 mM glucose	Glucose	0.09 g
2 mM MES	MES	2 ml 100 mM stock solution
Adjust pH to 5.8 with KOH, High temperature and high pressure sterilization for 20 min, room temperature preservation		

4. MMG solution

MMG solution (10 ml)		
15 mM $MgCl_2$	$MgCl_2$	1.5 ml 100 mM stock solution
4 mM MES	MES	0.4 ml 100 mM stock solution
0.6 M Mannitol	Mannitol	1.093 g
Adjust pH to 5.8 with KOH, High temperature and high pressure sterilization for 20 minutes, room temperature preservation		

Acknowledgments

This protocol is adapted from Wymer *et al*. (1999); Walter *et al*. (2004); Yoo *et al*. (2007) and Whang (2009).

References

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