Video Article

A Simple Method for Isolation of Soybean Protoplasts and Application to Transient Gene Expression Analyses

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Abstract

Soybean (*Glycine max* (*L.*) *Merr.*) is an important crop species and has become a legume model for the studies of genetic and biochemical pathways. Therefore, it is important to establish an efficient transient gene expression system in soybean. Here, we report a simple protocol for the preparation of soybean protoplasts and its application for transient functional analyses. We found that young unifoliate leaves from soybean seedlings resulted in large quantities of high quality protoplasts. By optimizing a PEG-calcium-mediated transformation method, we achieved high transformation efficiency using soybean unifoliate protoplasts. This system provides an efficient and versatile model for examination of complex regulatory and signaling mechanisms in live soybean cells and may help to better understand diverse cellular, developmental and physiological processes of legumes.

Video Link

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Introduction

Protoplasts are plant cells that have cell walls removed. As they maintain most of features and activities of plant cells, protoplasts are a good model system to observe and evaluate diverse cellular events, and are valuable tools to study somatic hybridisation[1] and plant regeneration[2]. Protoplasts have been also widely utilized for plant transformation[3,4,5], since cell walls would otherwise block the passage of DNA into the cell. Protoplasts possess some of the physiological responses and cellular processes of intact plants, hence offering fundamental value in basic research to study subcellular protein localization[6,7,8], protein-protein interactions[9,10], and promoter activity[11,12,13] in live cells.

The isolation of plant protoplasts was first reported in 1960[14] and the protocols for both isolation and transformation of protoplasts have been developed and optimized. A standard procedure of protoplast isolation involves the cutting of leaves and enzymatic digestion of cell walls, followed by separation of released protoplasts from non-digested tissue debris. Transformation strategies includes electroporation[15,16], microinjection[17,18], and polyethylene glycol-based (PEG)[4,5,19] methods. A wide range of species have been reported successful for protoplast isolation, including Citrus[20], Brassica[21], Solanaceae[22] and other ornamental plant families[23,24]. While diverse tissue types are used in various species, a system of transient expression in *Arabidopsis* mesophyll protoplast (TEAMP) isolated from leaves of the model plant *Arabidopsis thaliana* has been well established[25] and widely adopted to diverse applications.

Soybean (*Glycine max* (*L.*) *Merr.*) is one of the most important protein and oil crops[26]. Unlike *Arabidopsis* and rice, obtaining transgenic soybean plants is known to be rather difficult and low efficiency. *Agrobacterium tumefaciens*-mediated infiltration has been popularly used for transient gene expression studies in the epidermal cells in tobacco[27] and seedlings in *Arabidopsis*[28,29], whereas *Agrobacterium rhizogenes* has been used for transformation of hairy roots in soybean[30]. Virus-induced gene silencing approaches have been utilized for downregulation of target genes[31,32] and transient expression[33] in a systemic manner. Protoplasts provide a valuable and versatile alternative to these approaches. Protoplasts can be obtained from soybean's aboveground materials and allow quick and synchronized transgene expression. However, since the initial successful isolation of soybean protoplasts in the 1983[34], there have been limited reports on the application of protoplasts in soybean[35,36,37,38], primarily due to relatively low yields of soybean protoplasts.

Here, we describe a simple and efficient protocol for isolation of soybean protoplasts and its application for transient gene expression studies. Using young unifoliate leaves from soybean seedlings, we were able to obtain large quantities of vital protoplasts within a few hours. In addition, we have optimized a PEG-calcium-mediated transformation method that is simple and low cost to deliver DNA into soybean protoplasts with high efficiency.

Protocol

1. Growth of the plants

1. Sow 5 - 10 soybean seeds (Williams 82) in a 13 cm pot in the greenhouse under long-day conditions (16 h light at 1,500 µmol m^{-2} s^{-1}) at 25 °C on the custom soil mix for Soybean (the 1:1:1 ratio of soil, perlite and torpedo sand).

2. Preparation of Plasmid DNA

1. Using a sterile pipette tip or toothpick, pick a single colony or frozen glycerol stock of *E. coli* carrying the plasmid containing the gene of interest and inoculate it in 20 mL Luria-Bertani (LB) liquid medium with appropriate antibiotics in a 50 mL flask.
2. Incubate the flask at 37 °C overnight on a shaker. Collect the bacteria by centrifuging the suspension at 12,000 x g at room temperature for 5 min and discarding the supernatant. Extract and purify the plasmid following the manufacturer's procedure of a plasmid prep kit.

3. Protoplast isolation

1. Cut newly expanded unifoliate leaves from 10-day old soybean seedlings (**Figure 1**).
   NOTE: Selection of leaves at an appropriate developmental stage is the key to the success for soybean protoplast preparation. Only use just expanded leaves at early developmental stages. As leaves mature, cell walls become harder to digest.
2. With a fresh razor blade, remove the midrib from the unifoliate leaf and then cut the remains into 0.5-1 mm strips.
   NOTE: Two unifoliate leaves digested in 10 mL enzyme solution will give sufficient protoplasts for more than 10 transformations.
3. Using a pair of forceps, transfer the leaf strips immediately and gently into 10 mL of freshly prepared enzyme solution (**Table 1**) in a 15 mL tube. Vacuum infiltrate the leaf strips for 15 min at room temperature.
4. Incubate the leaf strips in the enzyme solution with gentle agitation (40 rpm) under low light for 4-6 h at room temperature. Ensure that the enzyme solution turns yellow-green as protoplasts are released. Check the enzyme/protoplasts solution under the microscope (X10).
   NOTE: The released protoplasts are spherical shaped, while the undigested cells have irregular or oval shape.
5. Transfer the 10 mL of enzyme/protoplast solution in a 50 mL tube by gently pouring and add 10 mL of W5 solution (**Table 1**) at room temperature to stop the digestion. Gently invert the tube a few times. Pour gently the enzyme/protoplasts solution on a clean 75 µm nylon mesh placed on top of a 50 mL tube to remove the undigested leaf tissues.
6. Centrifuge the flow-through enzyme/protoplasts solution at 100 x g in the 50 mL tube for 1-2 min at room temperature. Gently remove the supernatant using a 10 mL serological pipette without disturbing the protoplast pellet.
7. Resuspend the protoplasts and dilute to a concentration of 2 x 10^5 mL^{-1} in chilled W5 solution at 4 °C by counting protoplast number on a hemacytometer under the microscope (x10). Keep the protoplasts on ice for 30 min.
8. Centrifuge the suspension at 100 x g for 1-2 min at room temperature and gently remove the W5 solution using a 1 mL pipette without disturbing the protoplast pellet. Resuspend the protoplasts in MMG solution (**Table 1**) at a concentration of 2 x 10^5 mL^{-1} at room temperature.

4. Protoplast transformation

1. Make 100 µL aliquots of protoplasts (2 x 10^4 protoplasts at 2 x 10^5 mL^{-1}) in 1.5 mL low adhesion microcentrifuge tubes using uncut 200 µL pipette tips. Put one aliquot aside to serve as negative control. Add 10 µL of plasmid (10-20 µg) into each of the rest aliquot of protoplasts.
2. Slowly add 110 µL of freshly prepared PEG solution (**Table 1**) on the inner wall of the 1.5 mL microcentrifuge tube, and then gently invert and rotate the tube until the solution becomes homogeneous.
3. Incubate the transformation mixture at room temperature for 15 min.
4. To stop the transformation, slowly add 400 µL of W5 solution to the 1.5 mL tube at room temperature and gently invert the tube until the solution becomes homogeneous. Centrifuge the tube at 100 g for 1-2 min at room temperature and discard the supernatant using a pipette.
5. Add 1 mL of WI solution (**Table 1**) to the tube and resuspend by gently pipetting 1-2 times. Add 1 mL of 5% (vol/vol) sterile calf serum in each well of a 6-well tissue culture plate to coat the surface and prevent the protoplasts from sticking to the plate.
6. After a few seconds, discard the calf serum using a pipette. Transfer the resuspended protoplasts into a well of the culture plate. Cover the plate with a lid.

5. Protoplast incubation and harvesting

1. Incubate the protoplasts at room temperature for 1-2 days in the dark.
   NOTE: We found that two-days incubation yields stronger fluorescent protein signal in general compared to one-day incubation, and that the fluorescent protein signal may last for up to 3-4 days.
2. Transfer the protoplast solution to a 1.5 mL low adhesion microcentrifuge tube. Centrifuge the tube at 100 x g for 1-2 min at room temperature to harvest protoplasts. Remove the supernatant using a pipette and transfer 10 µL protoplasts onto a glass slide.
3. Observe fluorescent signal under fluorescence or confocal microscopy. Use non-transformed protoplasts as negative control (No signal should be observed).

Representative Results

Different organs of 10-day old soybeans were tested for protoplast preparation (**Figure 1**) and yields were observed under the microscope (**Figure 2**). Cell walls from hypocotyl and epicotyl were hardly digested, and some cells stayed attached to each other (**Figure 2B**, **2C**). In cotyledon (**Figure 2D**) and root (**Figure 2A**), cell walls were removed only in a small portion of the cells. In contrast, a large number of protoplasts were observed when unifoliate was used (**Figure 2E-G**). Unifoliate leaves at different developmental stages were further examined (**Figure 2H**). While both the unexpanded and just expanded unifoliate leaves resulted in high yields of protoplasts, the size of protoplasts from the just expanded unifoliate were more uniform (**Figure 2F**) than the unexpanded unifoliate (**Figure 2E**). For the fully expanded unifoliate, the cell walls were still intact in most of the cells (**Figure 2G**). We tested cell wall-digestion enzymes from three different manufacturers and obtained comparable results as described above. Based on these observations, we concluded that selection of plant materials was a crucial factor and that just expanded unifoliate leaves from young soybean seedlings were the best material for protoplast preparation.

A range of different amounts of plasmid DNA (0.1 µg, 1 µg, 5 µg and 20 µg) was tested for optimal transformation efficiency of soybean unifoliate protoplasts (**Figure 3A-D**). 20 µg plasmid DNA showed the highest transformation efficiency with more than 50% transformation rate (**Figure 3D**), while 0.1 µg showed the lowest (less than 1%) (**Figure 3A**). We obtained comparable transformation efficiency using different DNA purification kits from three manufacturers. This result suggests that larger amounts of plasmid DNA would greatly help to increase the transformation efficiency.

Figure 4 is confocal images of soybean protoplasts transformed with construct p2GWF7-E1, which expresses *GFP* fused to the legume-specific gene *E1* (Glyma.06G207800), driven by the CaMV *35S* promoter in the vector p2GWF7[39]. E1-GFP fusion protein shows nuclear localization in soybean protoplasts, which is consistent with a previous study using the Arabidopsis protoplast system[40]. Given that *E1* is a legume-specific gene, our result using the soybean protoplast system provides a conclusive insight in the subcellular localization of E1 protein.

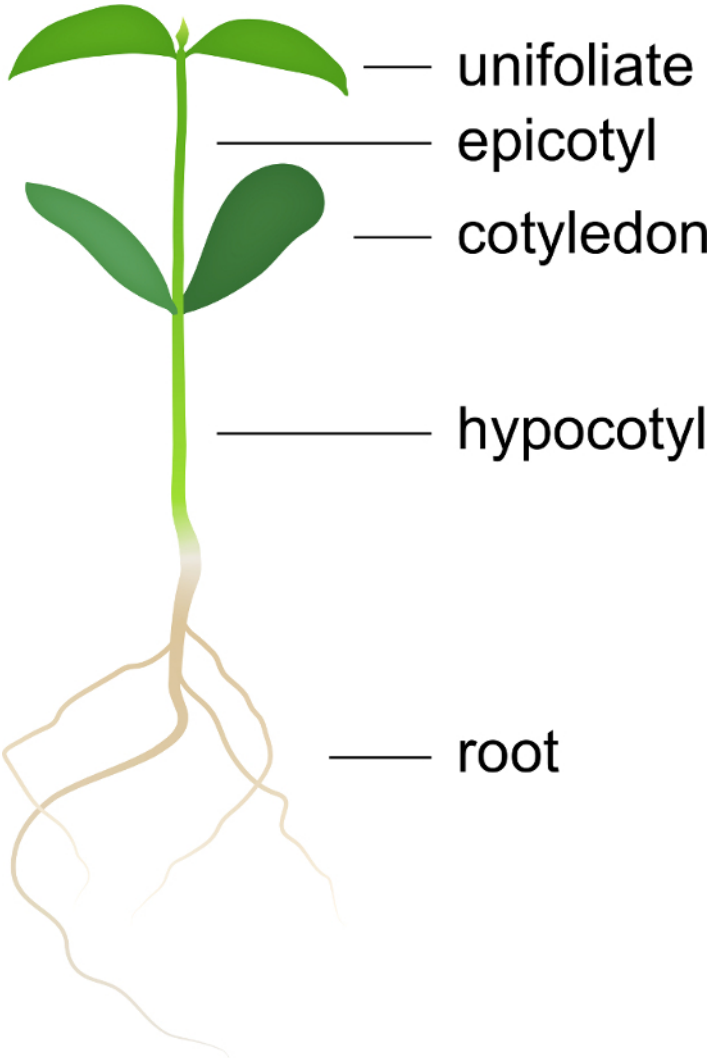

Figure 1. Illustration showing the organs of a soybean seedling that are tested for protoplast preparation in this study, including root, hypocotyl, cotyledon, epicotyl and unifoliate. After a pair of unifoliate leaves, soybean seedlings develop trifoliate leaves. Please click here to view a larger version of this figure.

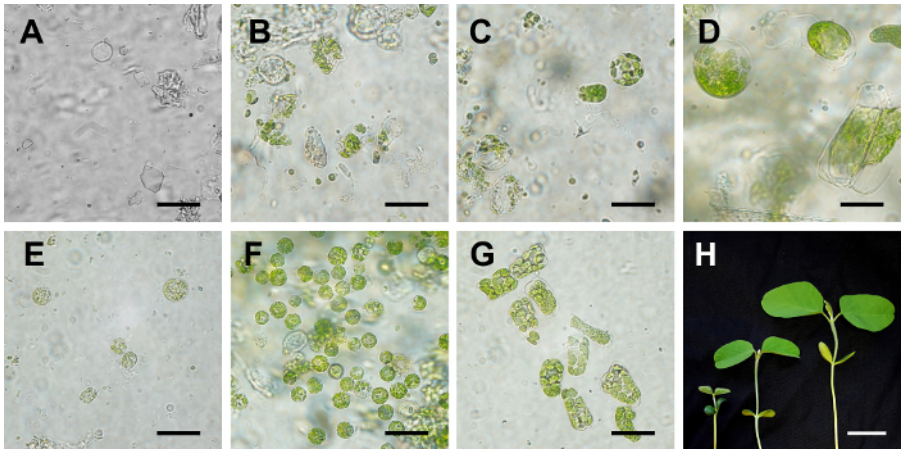

Figure 2. Protoplast cells prepared from different organs and developmental stages of soybean seedlings. (A-D) Cells prepared from different organs of 10-day old soybean seedlings: root (A), hypocotyl (B), epicotyl (C), cotyledon (D). (E-G) Protoplast cells prepared from unifoliate leaves at different developmental stages: unexpanded (E), just expanded (F), and fully expanded (G) unifoliate, corresponding to the soybean seedlings in (H) on the left, middle and right, respectively. The scale bar is 25 μm (A-G) or 25 mm (H). Please click here to view a larger version of this figure.

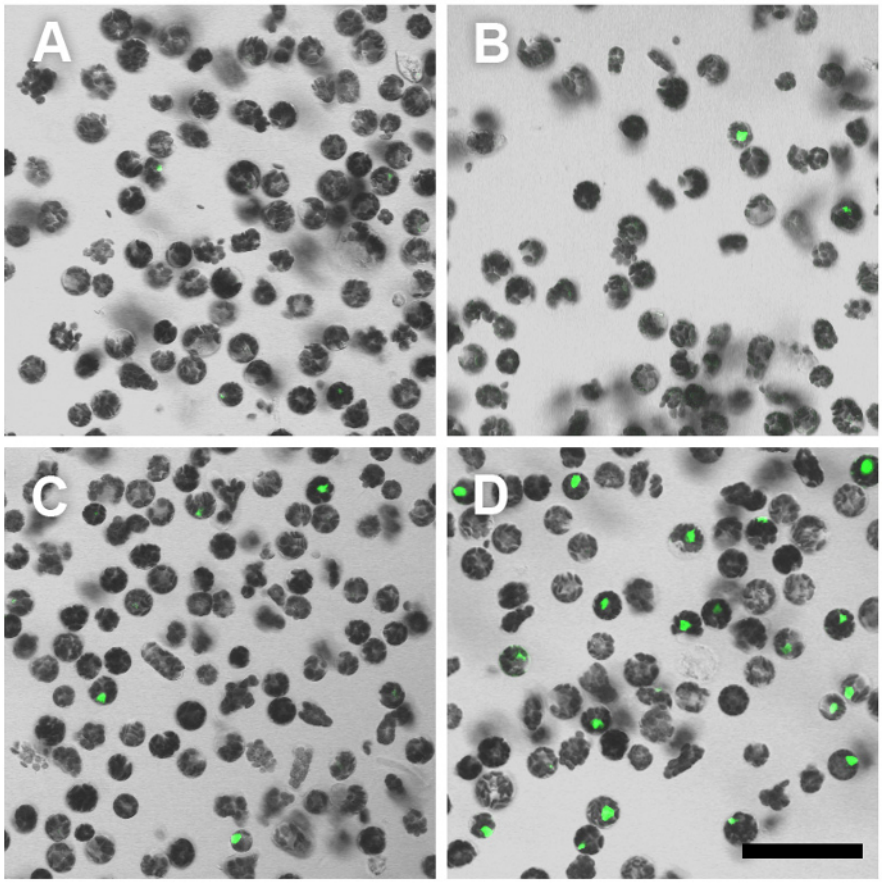

Figure 3. Confocal images showing transformation efficiency of soybean unifoliate protoplasts with different amounts of plasmid DNA. Images of GFP (represented in green) and bright field (grey) are merged. Protoplasts were transformed with different amounts of the plasmid p2GWF7-E1: 0.1 μg (A), 1 μg (B), 5 μg (C) and 20 μg (D). Fluorescence signal of GFP was monitored 24 hours after transformation under microscopy. The black scale bar is 50 μm. Please click here to view a larger version of this figure.

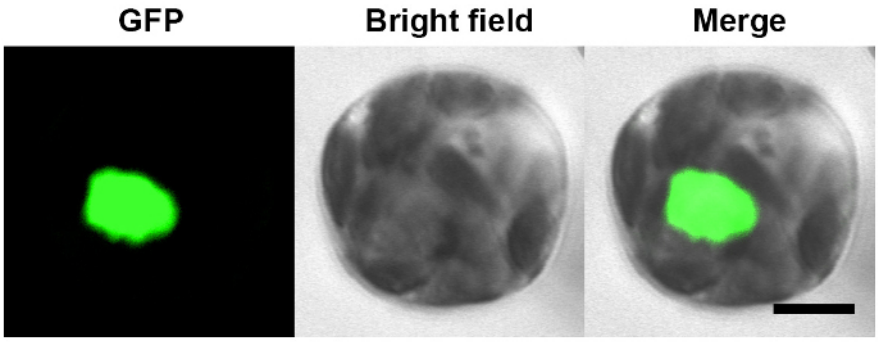

Figure 4. Confocal images showing the subcellular localization of E1-GFP in the nucleus of soybean unifoliate protoplasts. The plasmid p2GWF7-E1 was used for transformation. Images of GFP (represented in green) and bright field (grey) are merged. Fluorescence signal of GFP was monitored 24 hours after transformation. The black scale bar is 5 μm. Please click here to view a larger version of this figure.

Enzyme solution (freshly prepared)	
MES, pH 5.7	20mM
Cellulase CELF	2% (w/v)
Pectolyase Y-23	0.1% (w/v)
Mannitol	0.75 M
$CaCl_2$	0.2 mM
BSA	0.1% (w/v)
DTT	0.5 mM
W5 solution	
NaCl	154 mM
$CaCl_2$	125 mM
KCl	5 mM
MES, pH 5.7	2 mM
MMg solution	
MES, pH 5.7	4 mM
Mannitol	400 mM
$MgCl_2$	15 mM
PEG solution (freshly prepared)	
PEG4000	20% (w/v)
Mannitol	200 mM
$CaCl_2$	100 mM
WI solution	
MES, pH 5.7	4 mM
Mannitol	0.5 M
KCl	20 mM

Table 1. Solutions used for soybean protoplast isolation and transformation.

Discussion

This protocol for the isolation of soybean protoplasts and the application to transient expression studies has been thoroughly tested and works very well in our laboratory. The procedures are simple and easy and require ordinary equipment and minimum cost. Our protocol yields large quantities of uniform, high quality protoplasts compared to previously reported methods[34,35,36,37,38]. However, since there are many factors that affect protoplast yields and transformation rates, it is strongly recommended for researchers to optimize the conditions according to their experimental conditions, desirable results and materials used. While this system is extremely useful for examination of immediate regulatory and biochemical events in plant cells, it is not suitable for observation of long term cellular processes and events that occur at tissue or organismal levels.

We found that selection of vigorously growing plant materials at an ideal developmental stage was the most crucial factor in the soybean protoplast preparation. It determines not only yields of protoplasts, but quality that affects the subsequent DNA transformation. It is important to always grow soybean plants in a constant environment away from any stress, such as drought, flooding, extreme temperatures or pests. The highest protoplast yield and transformation efficiency can be achieved using just expanded unifoliate leaves from young seedlings. For beginners, it is suggested to use unifoliate leaves at different developmental stages and make a comparison to obtain the best results.

Protoplast/DNA ratio is an important factor for optimal transformation efficiency. It is usually best to start with the ratio of 2 x 10^4 protoplasts/10-20 µg DNA, but optimization of the ratio for individual constructs is recommended[25]. The use of high quality DNA obtained by a DNA purification kit is strongly recommended.

For the choice of vectors for transient expression in protoplasts, high-copy number and small-sized vectors are generally preferred. Although binary vectors for *Agrobacterium tumefaciens*-mediated transformation of plants can be used for protoplast transformation, the large size of these vectors may result in less optimal transformation efficiency. For GFP-fusion protein expression, we usually use vectors that do not possess a selection marker for plants and are thus relatively small (6-7kb), such as p2GWF7 and p2FGW7[39] (https://gateway.psb.ugent.be).

Multiple vectors can be used to transform protoplasts simultaneously. We had success in expressing BiFC vectors for testing protein-protein interactions, as well as multiple fluorescent proteins for organelle and subcellular labeling in soybean protoplasts. Furthermore, simplicity and large yields of our method offers an ideal system for examination of regulatory and biochemical events, gene-targeting vector design, isolation of transiently expressed proteins and protein complex, and purification of specific organelle such as nuclei, enabling further applications to emerging genomic and proteomic approaches.

Disclosures

The authors declare that they have no competing financial interests.

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