Transient expression of fluorescent fusion proteins in protoplasts of suspension cultured cells

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Transient expression of fluorescent fusion proteins in plant cells has dramatically facilitated our study of newly identified genes and proteins. This protocol details an *in vivo* transient expression system to study the subcellular localization and dynamic associations of plant proteins using protoplasts freshly prepared from *Arabidopsis* or tobacco BY-2 suspension cultured cells. The method relies on the transformation of DNA constructs into protoplasts via electroporation. The whole protocol is comprised of three major stages: protoplast generation and purification, transformation of DNA into protoplasts via electroporation and incubation of protoplasts for protein analysis. Similar to stably transformed cell lines, transformed protoplasts are compatible with protein localization studies, pharmaceutical drug treatment and western blot analysis. This protocol can be completed within 11–24 h from protoplast production to protein detection.

INTRODUCTION

The subcellular localization and dynamic interactions of proteins are important for their biological functions in plants. Transgenic *Arabidopsis* or tobacco plants and transgenic tobacco BY-2 cell lines stably expressing fluorescent reporter proteins have been widely used to study protein localization, protein function and organelle dynamics over decades[1–5]. For example, transgenic tobacco BY-2 cells expressing the YFP-BP-80 reporter have allowed the identification of multivesicular bodies as prevacuolar compartments (PVCs) and the demonstration of PVC vacuolation in response to wortmannin treatment in BY-2 cells[5]. However, it takes at least several months to generate and select these transgenic plants or cell lines after *Agrobacterium*-mediated transformation of plants or suspension cultured cells.

To speed up research, transient expression of fluorescent fusion proteins has also been successfully used to study protein localization and dynamics in plant cells. Depending on the cell type and methods of DNA introduction, several transient expression methods have been used with successful detection of the expressed proteins within a few days after transformation. These include *Agrobacterium* infiltration of tobacco epidermal cells, biolistics of cultured cells, and PEG- or electroporation-mediated transformation of protoplasts[6–9]. The latter method of using protoplasts has the advantage of short experimental duration and high transformation efficiency for various analyses, including protein localization, organelle dynamics and immunoprecipitation or western blot. Protoplasts used in these studies have been commonly generated from enzymatic digestion of mesophyll cells from either tobacco leaves or *Arabidopsis* young seedlings[6,7,10,11]. However, protoplast isolation in these studies requires a continuous supply of large amounts of tobacco plantlets or *Arabidopsis* seedlings grown in tissue culture conditions, which is a difficult and challenging task to meet for most plant laboratories. In contrast, fast-grown suspension cultured cells would conveniently provide relatively unlimited materials for protoplast isolation to be used in transient expression.

Thus, we have recently developed and tested a transient expression system using protoplasts isolated from *Arabidopsis thaliana* cell suspension cultures (ecotype Landsberg erecta) PSB-D and tobacco (*Nicotiana tabacum*) BY-2 suspension cultured cells, because these cells exhibit fast growth, are easy to culture and exhibit an absence of chloroplast-derived autofluorescence[12,13]. The application of this protocol requires four major stages: maintenance and subculture of suspension cultured cells, protoplast generation and purification, transformation of protoplasts by DNA via electroporation and study of the expressed fluorescent fusion proteins or XFP-labeled (labeled with variants of green fluorescent protein) organelles in protoplasts. More than 30% of the *Arabidopsis* protoplasts and 10% of the BY-2 protoplasts showed good fluorescent signals after transformation (**Fig. 1**). As a proof of principle, we used this protocol to test known fluorescent markers of the PVC for their localization and dynamics in protoplasts as compared to those previously obtained from transgenic tobacco BY-2 cell lines[1]. Identical results were obtained when the same fluorescent reporter proteins were either transiently expressed in protoplasts or stably expressed in BY-2 cells[4,5]. Furthermore, using this protocol, we have recently demonstrated that a member of the *Arabidopsis* cation diffusion facilitator family proteins, AtMTP11, localized to PVCs/multivesicular bodies because it colocalized with a fluorescent PVC marker when they were transiently coexpressed in BY-2 protoplasts[14]. This protocol is also compatible with pharmaceutical treatment and western blot analysis (see ANTICIPATED RESULTS). In addition, transformed protoplasts will be useful for studying organelle dynamics, *in vivo* protein–protein interaction via bimolecular fluorescence complementation or fluorescence resonance energy transfer and *in vitro* protein–protein interaction via immunoprecipitation[15–17]. Therefore, this protocol introduces a convenient, fast and reliable transient expression system for protein studies using protoplasts from plant suspension cultures.

Potential limitations of this protocol include (i) low transfection efficiency as compared to the PEG-mediated transformation of *Arabidopsis* mesophyll cells, where about 90% of the cells expressed the fusion proteins[18], (ii) limited application for expressing tissue- or organ-specific genes because of the undifferentiated feature of plant suspension cultured cells, and (iii) limited application for studying proteins of the photosynthetic pathway because these cultured cells lack green chloroplasts.

Experimental design

Selection of expression vector. Binary vectors are always difficult to manipulate owing to their large size (usually about 10–14 kb) and show low expression level and transformation efficiency in this protocol. Therefore, expression vectors of small size (about 3–6 kb) should be used in this protocol. We routinely use constructs derived from pBI221 under the control of cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase terminator (Clontech). **Figure 2** shows some of the constructs tested in this protocol.

DNA quality. DNA used for transient expression in this protocol is usually prepared by conventional DNA preparation methods in which bacteria are lysed by lysozyme-Triton solution, followed by plasmid DNA purification via phenol/chloroform extraction. Generally, purified DNA with an OD_{260}/OD_{280} value close to 1.8 is sufficient for this protocol. However, plasmid DNA with higher quality will further improve the transformation efficiency by 3–5% empirically. In this protocol, both CsCl-gradient centrifugation methods and the maxi-DNA preparation kit (Qiagen) were tested.

Quality of protoplasts. The growth conditions and growth rate for the suspension cultures of *Arabidopsis* and tobacco BY-2 cells are the most crucial factors that affect the quality of protoplasts and thus the success of subsequent DNA transformation and expression. In order to obtain large amounts of BY-2 cells, we have maintained and weekly subcultured the BY-2 cells in 50 ml cultures in 250 ml flasks at 23–24 °C in shakers at 120 r.p.m. as previously described[4,5,19,20]. Under these growth conditions, when the BY-2 cells are subcultured weekly according to the standard dilution ratio of 1:50 to 1:100 (see ref. 13) in a 50 ml volume, about 2–3 ml of compact cells can be collected at day 3 after subculture when the cells reach log phase. The BY-2 cells will thereafter grow into stationary phase; thus, the cultures will then contain cells of different stages[21]. To keep the BY-2 cells in the log phase all the time and to avoid mixing up of cells of different stages, we have thus modified the standard subculture protocol by subculturing the BY-2 cells every 3–4 d via 1:4 dilutions in the BY-2 MS medium. BY-2 cells grown under such conditions have a doubling time of 13–15 h at log phase, which is similar to that of the standard method[13]. In addition to providing relatively homogeneous BY-2 cells of log phase, such modification in subculture methods also results in a higher yield of BY-2 cells (10–15 ml of compact cells from the same 50 ml cultures) for protoplasting, and thus more qualified protoplasts for transformation. Furthermore, good protoplasts (>95% of cells) were generated within 2–3 h of enzyme digestion. The *Arabidopsis* cells are subcultured every 7 d in *Arabidopsis* MS medium.

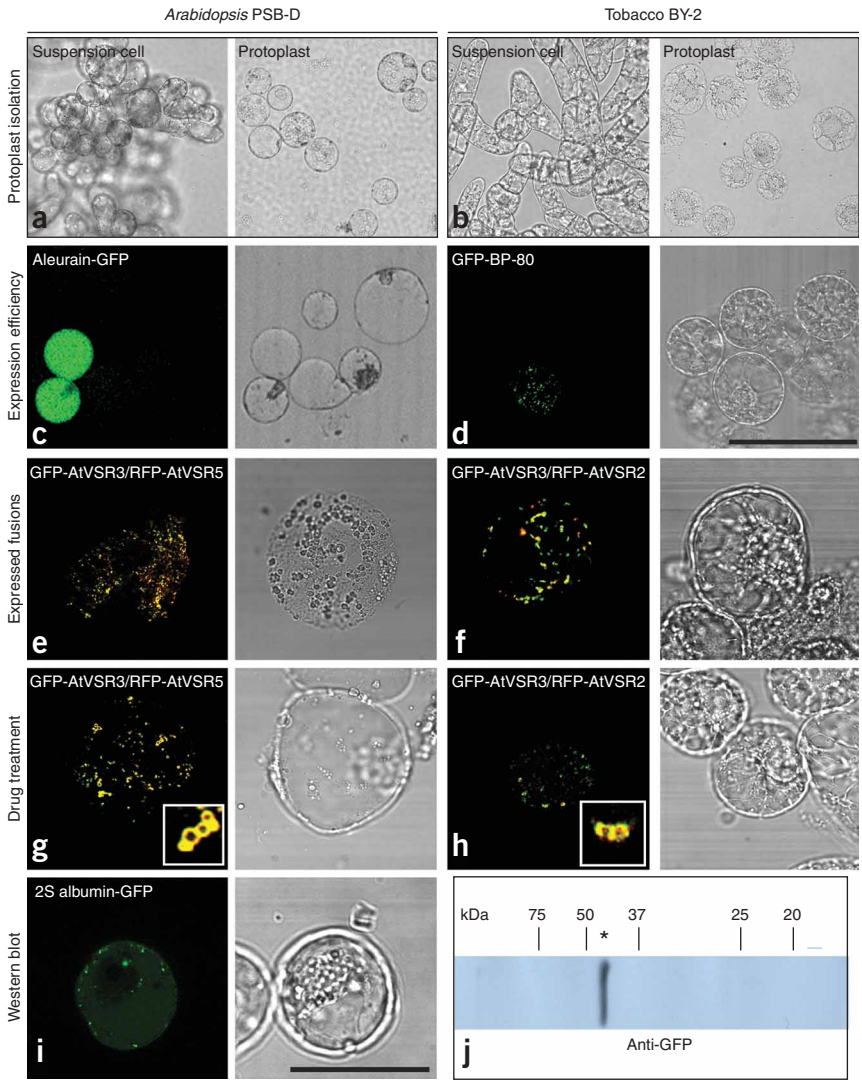

Figure 1 | Transient expression of fluorescent fusion proteins in *Arabidopsis* and BY-2 protoplasts. (**a**) Morphology of *Arabidopsis* suspension cultured cells and their protoplasts. (**b**) Morphology of tobacco BY-2 cells and their protoplasts. (**c**) Transient expression of a vacuolar fusion protein aleurain-GFP in *Arabidopsis* protoplasts, showing vacuolar localization. (**d**) Transient expression of a PVC fluorescent reporter GFP-BP-80 in BY-2 protoplasts. (**e**) Transient coexpression of two PVC reporters GFP-AtVSR3 (green) and mRFP-AtVSR5 (red) in *Arabidopsis* protoplasts, showing their colocalization. (**f**) Transient coexpression of two PVC reporters GFP-AtVSR3 (green) and mRFP-AtVSR2 (red) in BY-2 protoplasts, showing their colocalization. (**g**) *Arabidopsis* protoplasts coexpressing GFP-AtVSR3 and mRFP-AtVSR5 were treated with wortmannin (wort) at 16.5 μM for 1 h before image collection in living cells, showing their colocalization to wort-induced enlarged PVCs. (**h**) BY-2 protoplasts coexpressing GFP-AtVSR3 and mRFP-AtVSR2 were treated with wort at 16.5 μM for 1 h before image collection in living cells, showing their colocalization to wort-induced enlarged PVCs. (**i**) Transient expression of 2S albumin-GFP in *Arabidopsis* protoplasts, showing its punctate (PVC) and vacuolar localization. (**j**) Western blot analysis of GFP signals using anti-GFP on proteins extracted from *Arabidopsis* protoplasts expressing the 2S albumin-GFP construct. The asterisk indicates the position of the GFP fusion protein. The inset boxes in **g** and **h** are selective enlarged images of the wort-induced vacuolated PVCs containing the two XFP-AtVSR fusions. Scale bar, 100 μm (**a**–**d**), 50 μm (**e**–**i**).

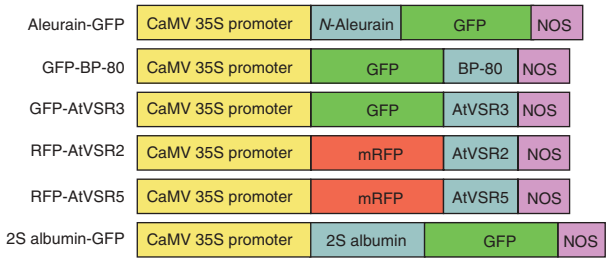

Figure 2 | Fluorescent fusion constructs used in this study. AtVSR2/3/5, transmembrane domain and cytoplasmic tail of AtVSR2/3/5 (*Arabidopsis* vacuolar sorting receptor 2/3/5); BP-80, transmembrane domain and cytoplasmic tail of the pea BP-80 (binding protein of 80 kDa); CaMV 35S promoter, cauliflower mosaic virus 35S promoter; GFP, green fluorescent protein; mRFP, monomeric red fluorescent protein; *N*-aleurain, N-terminal propeptide of barley aleurain; NOS, nopaline synthase terminator; 2S albumin, *Arabidopsis* full-length 2S albumin.

Time for the detection of transiently expressed proteins. The time needed for the transiently expressed fluorescent fusion proteins to reach detectable levels after electroporation will depend on the target protein. Some proteins are visible using a confocal microscope within 6 h after transformation, but an overnight incubation (12–16 h) is usually recommended.

MATERIALS

REAGENTS

- Murashige and Skoog Basal Salt Mixture (Sigma-Aldrich, cat. no. M5524)
- *myo*-Inositol (Sigma-Aldrich, cat. no. I3011)
- Thiamine hydrochloride (Sigma-Aldrich, cat. no. T4625)
- 2,4-Dichlorophenoxyacetic acid (Sigma-Aldrich, cat. no. D7299)
- KH_2PO_4 (Ajax Chemicals, cat. no. 391)
- Sucrose (Fluka, cat. no. 84097)
- Kinetin (Sigma-Aldrich, cat. no. K0753)
- 1-Naphthaleneacetic acid (Sigma-Aldrich, cat. no. N0640)
- Cellulase 'ONOZUKA' RS (Yakult Honsha, cat. no. L0011)
- Pectinase (Worthington, cat. no. LS004298)
- Driselase from *Basidiomycetes* sp (Sigma-Aldrich, cat. no. D9515)
- 2-(N-Morpholino) ethanesulfonic acid (MES) hydrate (Sigma-Aldrich, cat. no. M2933)
- HEPES (Sigma-Aldrich, cat. no. H3375)
- KCl (Riedel-de Haën, cat. no. 31248)
- $CaCl_2 \cdot 2H_2O$ (Ajax Chemicals, cat. no. 127)
- NH_4NO_3 (BDH, cat. no. 100303S)

EQUIPMENT

- Bench-top shaker set at 120 r.p.m. at room temperature (23 °C)
- Light-protected shaker set at 130 r.p.m. at 27 °C
- Incubators set at 26 and 27 °C
- 50 ml syringes
- 0.22 µm syringe filter
- 0.2 µm bottle-top filter (Nalgene, cat. no. 578816)
- Microscope slides and coverslips
- Sterile 1 ml top-cut blue pipette tips ▲ CRITICAL For eliminating impairment to protoplasts.
- Light microscope
- Pasteur pipette
- Petri dishes (85 mm × 15 mm and 30 mm × 15 mm)
- Laminar flow hood
- 50 ml conical tubes
- Centrifuge machine with swinging bucket rotor for 50 ml conical tubes (5810 Centrifuge; Eppendorf) ▲ CRITICAL For all washing steps, it is important to use the swinging bucket rotor, which allows the protoplast layer to float well on top.
- Confocal microscope
- Electroporation cuvettes with 0.4 cm gap (Bio-Rad, cat. no. 165-2088)
- Electroporation system (Gene Pulser Xcell serials; Bio-Rad, cat. no. 165-2661) ▲ CRITICAL The machine for electroporation should reach a voltage of 130 V and a capacitance of 1,000 µF.
- Peristaltic pump ▲ CRITICAL For pumping the solution out in the washing steps, the pump should work slowly and be manipulated manually; otherwise, protoplasts could be sucked into the Pasteur pipette.

REAGENT SETUP

***A. thaliana* cell suspension cultures (ecotype Landsberg erecta) PSB-D and tobacco (*N. tabacum*) BY-2 cells** *Arabidopsis* cells are grown in 250 ml flasks at 27 °C in light-protected shakers at 130 r.p.m. BY-2 cells are grown in 250 ml flasks at 23 °C in shakers at 120 r.p.m. *Arabidopsis* cells are subcultured in *Arabidopsis* MS medium once per week by transferring 5 ml of old cells into 45 ml of fresh *Arabidopsis* MS medium. Tobacco BY-2 cells are subcultured in BY-2 MS medium twice per week by transferring 10 ml of old cells into 40 ml of fresh BY-2 MS medium. ▲ CRITICAL BY-2 cells should be subcultured every 3–4 d to keep them growing actively at log phase, which is critical for good protoplasting and gene expression[12].

BY-2 MS medium 4.3 g liter^{-1} Murashige and Skoog Basal Salt Mixture, 100 mg liter^{-1} *myo*-inositol, 1 mg liter^{-1} thiamine hydrochloride, 0.2 mg liter^{-1} 2,4-dichlorophenoxyacetic acid, 255 mg liter^{-1} KH_2PO_4 and 30 g liter^{-1} sucrose, pH 5.0 (with KOH). BY-2 MS medium can be stored at room temperature (23 °C) for up to 3 months.

***Arabidopsis* MS medium** 4.3 g liter^{-1} Murashige and Skoog Basal Salt Mixture, 100 mg liter^{-1} *myo*-inositol, 0.4 mg liter^{-1} thiamine hydrochloride, 50 µg liter^{-1} kinetin, 800 µg liter^{-1} 1-naphthaleneacetic acid and 30 g liter^{-1} sucrose, pH 5.7 (with KOH). ▲ CRITICAL *myo*-Inositol and thiamine hydrochloride should be sterilized by filtration through a 0.22 µm syringe filter first and then added to the autoclaved MS medium in a laminar flow hood. *Arabidopsis* MS medium can be stored at room temperature (23 °C) for up to 3 months.

Enzyme solution 1% (wt/vol) cellulase 'ONOZUKA' RS, 0.05% pectinase and 0.2% driselase from *Basidiomycetes* sp in protoplast culture medium. The enzyme solution can be dissolved in protoplast culture medium at 4 °C overnight or alternatively it can be dissolved at 30 °C with shaking at 130 r.p.m. for 1 h. Finally, the enzyme solution should be clear light brown. Filter the enzyme solution through a 0.22 µm syringe filter device into a 50 ml conical tube. ▲ CRITICAL The enzyme solution should be prepared fresh.

Electroporation buffer 0.4 M sucrose (13.7%), 2.4 g liter^{-1} HEPES, 6 g liter^{-1} KCl and 600 mg liter^{-1} $CaCl_2 \cdot 2H_2O$, pH 7.2 (with KOH). Electroporation buffer can be stored at room temperature (23 °C) for up to 3 months. ▲ CRITICAL The concentration of sucrose is critical to float the healthy protoplasts on the top layer during the protoplast washing steps.

Protoplast culture medium 4.3 g liter^{-1} MS salts, 0.4 M sucrose (13.7%), 500 mg liter^{-1} MES hydrate, 750 mg liter^{-1} $CaCl_2 \cdot 2H_2O$ and 250 mg liter^{-1} NH_4NO_3, pH 5.7 (with KOH). Protoplast culture medium can be stored at room temperature (23 °C) for up to 3 months. ▲ CRITICAL Electroporation buffer and protoplast culture medium should be sterilized by filtration through a 0.2 µm bottle-top filter in a laminar flow hood because of the presence of sucrose in the media.

PROCEDURE

Preparation of protoplasts for transient expression ● TIMING 2.5–4 h

1| Prepare 50 ml of enzyme solution (see REAGENT SETUP) in protoplast culture medium (see REAGENT SETUP) in an 85 ml flask covered with a foil and dissolve the enzymes slowly overnight at 4 °C. Generally, a 50 ml enzyme solution is enough to prepare protoplasts sufficient for up to ten transformations.

▲ CRITICAL STEP After incubation at 4 °C overnight, the enzyme solution should be brought back to room temperature (23 °C) before incubation with the plant cells to avoid cold stress.
▲ CRITICAL STEP All the following steps should be carried out in a laminar flow hood.

2| Transfer 50 ml of 3- to 5-d-old *Arabidopsis* cells or 2- to 3-d-old BY-2 cells into 50 ml conical tubes in a laminar flow hood.

3| Pellet the cells by centrifuging at about 100*g* for 2 min at room temperature and remove the supernatant.

4| Sterilize the enzyme solution by passing through a 0.22 μm syringe filter using a 50 ml syringe and transfer 40–45 ml of enzyme solution into each conical tube containing 5–10 ml of compact cells. Mix slowly by inverting the tube several times until no obvious cell pellets can be observed.

5| Aliquot the 50 ml of well-mixed cells into three Petri dishes (85 mm × 15 mm) and incubate them in a shaker set at 65 r.p.m. at 25 °C for 2.5–3 h for BY-2 cells or 27 °C for 1.5–2 h for *Arabidopsis* cells.
▲ CRITICAL STEP Check protoplasts under a light microscope every 30 min until more than 95% of the cells form individual and round-shaped protoplasts.

Protoplast washing using electroporation buffer ● TIMING 1 h

6| Transfer the protoplasts into new 50 ml conical tubes and centrifuge at room temperature for 15 min at 80*g* using a swinging bucket rotor.
▲ CRITICAL STEP The protoplasts in good condition will float to the top after centrifugation. Poor floating of protoplasts represents bad condition of protoplasts, which will decrease the transformation efficiency. All centrifugation steps should be carried out without deceleration to prevent the top protoplast layer from being disturbed. Hold the tube carefully after the centrifugation is completed.

7| Insert a Pasteur pipette through the floating protoplasts layer in hood and suck out the underlying solution by the Pasteur pipette connected with a peristaltic pump until the floating protoplasts become close to bottom.
▲ CRITICAL STEP Before switching on the pump, use the Pasteur pipette to open a small area on top to prevent the protoplasts from being absorbed into the Pasteur pipette during the insertion step. Insert the Pasteur pipette upright to the bottom and switch on the pump. Finally, pull up the Pasteur pipette quickly and then switch off the pump.

8| Add 35 ml of electroporation buffer into the protoplasts and mix them gently, and centrifuge again at 80*g* for 10 min.

9| Repeat the washing Steps 7 and 8 twice.
▲ CRITICAL STEP These washes remove traces of enzyme solution to reduce their negative effects on DNA transfection efficiency and to keep the good protoplasts on the top. The underlying solution should become clear without visible pellets. Otherwise, repeat the washes.

Transfer DNA into protoplasts via electroporation ● TIMING 1.5–2 h

10| Resuspend the protoplasts gently using electroporation buffer in an appropriate volume to obtain 2–5 × 10^6 protoplasts per ml based on counting with a hemacytometer. Generally, this can be achieved by resuspending the protoplasts in two volumes of electroporation buffer. For example, 2.5 ml of healthy floating protoplasts can be diluted with 5 ml of electroporation buffer.

11| Aliquot 500 μl of the resuspended protoplasts into 0.4 cm gap electroporation cuvettes using top-cut blue pipette tips. Such cut tips with larger mouths can avoid shearing of the protoplasts.

12| Mix 40 μg plasmid DNA with electroporation buffer to a final volume of 100 μl.
▲ CRITICAL STEP The pH 7.2 of electroporation buffer is not favorable to plant cells, so it is wise to prepare DNA in advance so that you can mix DNA with protoplasts immediately after the washing steps. Transformation efficiency will be increased if the amount of plasmid DNA used is increased from 10 to 40 μg, but a DNA amount higher than 40 μg will not increase the transient transfection efficiency empirically.

13| Mix the 100 μl plasmid DNA and 500 μl protoplasts gently by flipping container on side and incubate for 5–10 min at room temperature.

14| Electroporate the protoplasts at 130 V and 1,000 μF for one pulse. The pulse time will result in a range from 25 to 50 ms.
! CAUTION The voltage used here is harmful; safety-guaranteed electroporation instrument is required.

15| Incubate the electroporated protoplasts at room temperature for 20–30 min without moving.
▲ CRITICAL STEP Because the protoplasts are very fragile after the electroporation, move out the cuvettes gently from the machine and put them aside in the laminar flow hood during the incubation period. Generally, the protoplasts will float up to the top after

this incubation step, and the final volume of the floating protoplasts should be in the range from one-fifth to one-half of the total volume, otherwise the DNA transfection efficiency will be reduced.

16| Remove 200–300 µl of the underlying electroporation buffer from the cuvettes. Too much leftover electroporation buffer will change the pH of the protoplast culture medium and affect the cell growth condition and thus the protein expression.

17| Add 1 ml of protoplast culture medium to each cuvette and pour the protoplast suspension into a small Petri dish of 30 mm × 15 mm.

18| Add another 1 ml of the protoplast culture medium to wash the cuvette and pour the solution together into the previous Petri dish in Step 17.

Incubation of electroporated protoplasts ● TIMING 6–18 h

19| Incubate the protoplasts at 26 °C for BY-2 cells and at 27 °C for *Arabidopsis* cells for 6–18 h before observation for fluorescent signals or protein extraction for western blot or immunoprecipitation analysis.

! CAUTION The expression ratio and signal intensity are also gene dependent.

? TROUBLESHOOTING

● TIMING

Step 1, overnight or 1 h
Steps 2–4, 10 min
Step 5, 1.5–3 h
Steps 6–9, 1 h
Steps 10–13, 1 h
Steps 14–18, 25–40 min
Steps 19, 6–18 h

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Problem	Possible reason	Solution
Poor floating of BY-2 protoplasts to top during the washing steps	Poor condition of BY-2 cells	Subculture BY-2 cell twice per week to maintain their active growth at log phase
Low expression ratio and level	Poor condition of initial plant culture cells	Subculture BY-2 cell twice per week to maintain their active growth at log phase
	Poor digestion of protoplast	Wash protoplasts only when more than 95% cells become protoplasts
	Protoplasts unclean before electroporation	Repeat the washing steps for protoplasts with electroporation buffer until the medium becomes clear
	Poor DNA quality	Prepare plasmid DNA using CsCl-gradient centrifugation methods

ANTICIPATED RESULTS

This protocol describes a fast transient expression method using protoplasts derived from suspension cultured cells, which can be used for studying protein localization, organelle dynamics and protein–protein interaction in plant cells. Transient expression of fluorescent fusion proteins can be achieved by transferring DNA constructs into protoplasts via electroporation. These constructs are usually under the control of the CaMV 35S promoter in pBI221-derived expression vectors. Single or pairs of XFP fusions can be simultaneously expressed in the same cells. Subsequently, subcellular localization, drug treatment and western blot studies can be carried out on transformed protoplasts. More than 95% of *Arabidopsis* and BY-2 cells produce good protoplasts upon enzyme digestion (**Fig. 1a**,**b**). About 30% of the *Arabidopsis* protoplasts and 10% of the BY-2 protoplasts showed good fluorescent signals at 12 h after electroporation (**Fig. 1c**,**d**).

As a proof of principle, we transferred and tested pairs of XFP fusion proteins with vacuolar sorting receptor (VSR) that were known to locate to PVCs in stably transformed tobacco BY-2 cells[1,3]. When pairs of green fluorescent protein (GFP)-AtVSR3 and monomeric red fluorescent protein (mRFP)-AtVSR5 were transiently expressed together in either *Arabidopsis* protoplasts or BY-2

protoplasts, they largely colocalized to punctate PVC organelles (**Fig. 1e**,**f**). Similarly, organelles marked by these two fusions in both *Arabidopsis* and BY-2 protoplasts formed small vacuoles in response to wortmannin treatment (**Fig. 1g**,**h**), indicating their PVC nature, as demonstrated previously in transgenic tobacco BY-2 cells[4,5]. Thus, identical results were obtained when the same fluorescent PVC markers were transiently expressed in either BY-2 or *Arabidopsis* protoplasts as compared to those of stably transformed BY-2 cells. *Arabidopsis* protoplasts transiently expressing PVC/vacuole-localized 2S albumin-GFP (**Fig. 1i**) were also subjected to protein extraction and western blot analysis using GFP antibody (**Fig. 1j**) where a 48 kDa band representing the full-length GFP-2S albumin fusion was detected. Thus, protein detection and protein interaction via immunoprecipitation is also feasible in protoplasts after transient expression.

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