Chapter 2

Expression of Epitope-Tagged Proteins in Plants

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Abstract

Although immunoelectron microscopy is a powerful tool for visualizing the subcellular localization of target proteins, it is difficult to obtain and purify the specific antibodies required for this method. Instead of raising antibodies against individual target proteins, the use of transgenic plants expressing epitope-tagged proteins and commercially available antibodies simplifies the subcellular localization of target proteins. In this chapter, an improved method for producing transgenic plants that express epitope-tagged proteins and can be used for immunoelectron microscopic analysis is described.

Key words: Epitope tag, transformation, Arabidopsis thaliana, Agrobacterium tumefaciens.

1. Introduction

Agrobacterium tumefaciens is a bacterium that causes tumors (crown gall) in dicotyledonous plants by transferring a small segment of its own DNA (“transfer DNA” [T-DNA] in a Ti plasmid) into the plant’s genome. It is therefore possible to transfer a gene of interest into the genomic DNA of a dicot by subcloning the gene into the T-DNA of Agrobacterium (between a pair of border sequences for gene transfer). Although this method of obtaining transgenic plants is applicable for various plant species including some monocots, it is complicated and requires careful handling for the establishment of the callus and the regeneration of the plant from transformed cells (1, 2). In order to transform Arabidopsis thaliana, a model plant used to study the development and stress responses of plants, a simplified,
The Agrobacterium-mediated transformation method termed “floral dipping” (3) is commonly used.

In order to instantly identify the subcellular localization of proteins in plant cells, a fluorescent protein such as the green fluorescent protein (GFP) is fused to the N-terminus or C-terminus of the protein under study, and the fusion construct is then stably or transiently expressed in plant cells. The use of such fluorescent constructs enables direct subcellular localization of the GFP-tagged protein by confocal microscopy. Although GFP-based imaging is a simple and rapid tool, it has some limitations in terms of resolution and sensitivity. If the expression level of the mature target protein is low or if the protein is localized in small amounts in different regions, detection is difficult. In addition, GFP (27 kDa) has a tendency to localize at the nucleus.

In comparison, immunohistochemistry and immunoelectron microscopy, which involve the use of a specific antibody against native endogenous proteins, are more efficient techniques for studying the subcellular localization of proteins, even though their working procedures are more complex. For these techniques, recombinant proteins containing the entire or partial amino acid sequence of the target protein are purified, and antibodies (IgG) against these proteins are raised in animals such as mice or rabbits. A variety of monoclonal and polyclonal antibodies against both soluble and membrane proteins of human or animal origin have recently become commercially available. Unfortunately, such “antibody pools” are not available for research on plant cells.

Epitopes, such as FLAG, c-Myc, and polyhistidine, are supposed to be small enough (composed by 6–10 amino acids) to preserve the structure and the subcellular localization of epitope-tagged proteins resulting in the same localization as the corresponding endogenous proteins. Highly specific monoclonal antibodies against epitopes are commercially available, meaning that expression of epitope-tagged proteins is an alternative method for investigating the subcellular localization of target proteins by immunohistochemistry or immunoelectron microscopy without raising any specific antibodies. A further advantage of immunoelectron microscopy of transgenic cells expressing epitope-tagged proteins is the higher detection sensitivity based on the overexpression of the target protein, the high-affinity binding of antibodies against epitope tags, and the high-resolution localization by electron microscopy. This chapter provides a modified method to express epitope-tagged proteins in A. thaliana by Agrobacterium-mediated plant transformation.
2. Materials
(see Note 1)

2.1. Cultivation of Arabidopsis for Agrobacterium-Mediated Transformation

1. Seeds of *A. thaliana* (ecotype Columbia-0 [Col-0]) (see Note 2)
2. Mixed soil (Potting soil:sand:pumice = 7:2:1 or potting soil:vermiculite:perlite = 4:3:2) (see Note 3)
3. Plastic pots for plant growth (70–80 mm diameter, 70–90 mm tall)
4. Spatula
5. Plant growth chamber with air conditioner and illumination controller (see Note 4)
6. Micropipettes and disposable tips (for 20–200 and 100–1,000 μL) (Eppendorf AG, Hamburg, Germany)
7. 10 mL of double-distilled H₂O (autoclaved)
8. 1.5-mL microfuge tube (Eppendorf AG, Hamburg, Germany)
9. Transparent film. (A plastic wrap for cooking is suitable)

2.2. Infection with *A. tumefaciens*

1. Binary plasmid with a cDNA encoding an epitope-tagged protein to be expressed and a plant selection marker such as hygromycin resistance (see Note 5)
2. Electro-competent cells of *A. tumefaciens* host strain (such as a strain of LBA4404 [Takara Bio Inc.; Otsu, Japan]) (see Note 6)
3. Gene Pulser II electroporation system (Bio-Rad; Hercules, CA, USA)
4. 0.2 cm gap width electroporation cuvettes (Bio-Rad; Hercules, CA, USA)
5. YEB medium: 5 g/L Bacto peptone (BD, Sparks, MD), 5 g/L beef extract, 1 g/L yeast extract (BD), 5 g/L sucrose, and 2 mM MgSO₄. Adjust the pH to 7.4 with 0.5 M NaOH, then sterilize by autoclaving at 121°C for 15 min.
6. LB (Luria Bertani) selection medium: 10 g/L Bacto tryptone, 5 g/L yeast extract, 5 g/L NaCl. Adjust the pH to 7.4 with 0.5 M NaOH, then sterilize by autoclaving at 121°C for 15 min. After the medium has cooled, add the corresponding antibiotics for transformant selection. (for hygromycin-resistant marker, use 50 μg/mL hygromycin for the selection of *Agrobacterium* colonies and transformed plants containing plasmid or T-DNA)
7. LB selection plates: Prepare LB medium and add 1% agar before autoclaving. After the medium has cooled, add antibiotic for the selection of transformants and pour the medium into plastic petri dishes (90 mm diameter; approximately 30 mL/dish) on a clean bench.

8. Infiltration medium: Mix 4.3 g Murashige and Skoog salt mixture (Sigma, St. Louis, MO), 0.12 g Gamborg’s B5 vitamin mixture (Sigma, St. Louis, MO), 50 g sucrose, 0.5 g 2-(N-morpholino)ethanesulfonic acid (MES), and distilled water for a final volume of 1 L. Sterilize by autoclaving at 121°C for 15 min. Before using the medium for infection, add 10 μL/L 1 mg/mL benzylaminopurine in dimethyl sulfoxide [DMSO] and 10 μL/L Silwet L-77 (GE Silicones, WV, USA)

9. Tall beaker, 200 mL (60 mm diameter, 115 mm tall).

10. Paper towels

11. 100–200 mL of 70% ethanol

12. Disposable, sterilized plastic petri dishes (90 mm diameter)

13. 15-mL test tubes (BD Falcon™ Conical Tubes, BD, Franklin Lakes, NJ)

14. Rotary shaker for culturing

15. Incubator for bacterial culture (at 30°C, keep inside dark)

16. Flat trays without drainage holes and clear plastic domes (Humi-dome, Hummert Intl., St. Louis, MO)

17. Vacuum pump

18. Vacuum desiccator

19. 100-mL and 1-L Erlenmeyer (conical) flasks

20. Centrifuge (up to 6,000 × g) (Eppendorf AG, Hamburg, Germany)

21. 50-mL disposable tubes (BD Falcon™ Conical Tubes, BD, Franklin Lakes, NJ)

2.3. Seed Collection

1. Letter-size envelopes

2. Stainless steel tea filters (two to three pieces) to separate seeds from dried siliques and stems (diameter of mesh: 1–2 mm)

2.4. Selection of Putative Transformants

1. Selection medium: Mix 2.15 g Murashige and Skoog salt mixture, 100 mg myo-inositol, 10 g sucrose, 10 mL of 5% MES-KOH (pH 5.8), 3 g Gelrite (Wako, Japan) or 10 g agar, and distilled water for a final volume of 1 L. Sterilize by autoclaving at 121°C for 15 min. After the medium has cooled, add antibiotic for the selection of transformants and 100 μg/mL carbenicillin to sterilize the Agrobacterium.
Pour the medium into plastic petri dishes (90 mm diameter, 18 mm depth; approximately 30 mL/dish) on a clean bench.

2. 200 mL of double-distilled H₂O (autoclaved)
3. 10 mL of 0.1% sodium dodecyl sulfate (SDS)
4. 100 mL of bleach (sodium hypochlorite solution)
5. 0.1% sterile agarose solution: 0.1 g/L electrophoresis-grade agarose (Sigma, St. Louis, MO). Sterilize by autoclaving at 121°C for 15 min.

3. Methods

3.1. Cultivation of Arabidopsis for Agrobacterium-Mediated Transformation

1. Transfer 50–100 Arabidopsis seeds into a 1.5-mL microfuge tube using a spatula or by tapping.
2. Add 1 mL of water and tap the tube until the seeds separate, sink into the water, and swell. Store the tube in the dark at 4°C for 2 days (in a cold room or refrigerator) so that the seeds acquire the competence to germinate.
3. Pick up the seeds using a micropipette (100–1,000 μL) with a disposable tip and sow them in the moistened soil mixture in a plastic pot.
4. To prevent the seeds and surface of soil from drying out, cover the top of the pot with a petri dish or transparent film.
5. Grow the seedlings in a chamber with short-day conditions (8 h of daylight and 16 h of darkness). Normally, most of the seeds germinate within 3–5 days. After germination, remove the dish or film covering the top of the pot.
6. Two weeks after sowing, transplant individual seedlings into new pots.
7. Allow the seedlings to grow under short-day conditions until the rosette leaves are well developed (i.e., till they nearly cover the top of the pot Fig. 2.1 A). This may require 4–8 weeks. Usually, dipping of 6–8 plants yield an adequate number of transformants.
8. Transfer the pots into a chamber with long-day conditions (16 h of daylight and 8 h of darkness) to initiate the bolting process.
9. To obtain a greater number of floral buds per plant, cut the apical region of the inflorescence when bolting is complete in most of the plants. This process promotes the synchronized emergence of multiple secondary bolts (see Note 7 and Fig. 2.1 B).
Fig. 2.1. *Arabidopsis* plants for *Agrobacterium*-mediated transformation. (A) A plant grown under short-day conditions. Rosette leaves (more than 20 pieces) are developed. Well-established roots support the soil from falling into the dipping bath when the plant is turned upside down. (B) A plant with multiple secondary bolts. Arrowhead indicates the clipped position of an inflorescent stem. (C) Upside down placement of a plant on a tall beaker (100 mL) for dipping. Diameter of the beaker is slightly smaller than the diameter of the pot to ensure that the pot is supported and does not fall into the beaker. Arrowhead indicates the approximate position of the surface of dipping solution (5–10 mm lower from the edge of beaker).

### 3.2. Infection with *A. tumefaciens*

1. Dissolve 40 μL of electro-competent *A. tumefaciens* cells in a microfuge tube on ice and add 1 μL (50–200 ng) of a binary plasmid construct.

2. Transfer the DNA cell mixture to an ice-cold electroporation cuvette for the electroporation. Electroporate the plasmids into the cells using a resistance of 200 Ω, a capacitance of 25 μF and a voltage of 2.5 V (see Note 8).

3. Immediately add 1 mL of YEB medium, mix by pipetting, and transfer to a 15 mL test tube. Incubate at 30°C, for 1 h on a rotary shaker rotating at 100–150 rpm.
4. Spread the electroporated cells onto a LB selection plate. To obtain a single colony of transformed *A. tumefaciens*, drop the inoculum of a differentiated portion (10–200 μm) onto 5–10 plates. After incubation at 30°C for 2 days in the dark, a number of colonies will be obtained.

5. When many unopened (immature) floral buds are observed, inoculate the *A. tumefaciens* transformed with the binary vector into 10 mL of LB selection medium in a 100-mL Erlenmeyer (conical) flask and incubate at 30°C overnight in the dark.

6. Dilute the overnight *Agrobacterium* culture with 300 mL of LB selection medium (1:100) in a 1-L Erlenmeyer flask. Culture at 30°C for 14–20 h until the cells reach the stationary phase (OD$_{600}$ = 1.2–1.5).

7. Harvest the *A. tumefaciens* cells by centrifugation at 5,000–6,000×g for 10 min at room temperature and resuspend them in infiltration medium to obtain an OD$_{600}$ of 0.8. The suspension termed “dipping solution” is used for the infection (see Note 9).

8. Pour 200–220 mL of the *Agrobacterium* suspension, dipping solution, into 200-mL-tall beakers. The beakers must have a small diameter to ensure that the pots are supported and do not fall into the beakers. Each “dipping bath” can be used up to five times.

9. Remove the flowers (opened buds) and siliques from the plants. Turn the plant upside down and gently submerge the aerial part (including the rosette leaves) into the dipping solution for 10–15 min (see Note 10 and Fig. 2.1C).

10. Remove the plants from the dipping bath. Gently shake or wipe off the dipping solution remaining on the aerial part of the plant using a paper towel.

11. Place the plants in a plastic tray separating each plant from its neighbors to avoid contamination. Cover the plants with a plastic dome to maintain humidity because dipped plants are sensitive to drought owing to the hyperosmotic stress induced by the dipping solution. Place the plants under conditions of low light intensity to promote the breeding and infection of *Agrobacterium*.

12. Remove the plastic dome 2 days after infection. To avoid the withering of dipped plants, do not water them until the soil is relatively (not completely) dry. Usually, watering can be resumed within 1 week.

13. Allow the plants to grow for 3–5 weeks until the siliques developed from the dipped buds have matured (i.e., they appear brown and dry).
3.3. Seed Collection

1. Carefully harvest the aerial parts of the plants (except the rosette leaves) and transfer them into letter-size envelopes. Plants that have the same genotype and have been dipped in the solution containing the same cDNA construct can be put into a single envelope. Store the plants and seeds at room temperature until they are completely dry.

2. Release the seeds from the siliques by gently rubbing fingers over the envelope. Remove the stems and the outer portions of the siliques by filtering them through a stainless steel tea filter and collect the seeds on a clean piece of paper. Transfer the seeds into microfuge tubes and store them at 4°C under desiccation conditions for further use.

3.4. Selection of Putative Transformants

1. Transfer 2,000–3,000 seeds obtained from the dipped plants into a microfuge tube by using a spatula or by tapping. Add 600 μL of water and tap the tube until the seeds separate, sink into the water, and swell sufficiently.

2. Add 20 μL of 0.1% SDS and mix briefly by inverting the tube. Add 400 μL of bleach to sterilize the seed surface and remove any contaminating fungi and bacteria (see Note 11). Mix well for 10 min by inverting the tube or by gently shaking it on a rotary shaker.

3. Let the tube stand on a rack to allow the seeds to settle to the bottom. Remove the sterilization buffer and rinse the seeds five to seven times with 1 mL of sterilized double-distilled H₂O to remove the remaining sodium hypochlorite.

4. Suspend the seeds in 1 mL of 0.1% sterile agarose solution and plate this suspension on selection medium (500–600 seeds per plate). Place the plates in the dark at 4°C for 2 days (in a cold room or refrigerator) so that the seeds acquire the competence to germinate.

5. Transfer the plate to a growth chamber with long-day conditions. Within 2 weeks, the non-transformed plants (>90%) die (i.e., they turn white), because of the effect of the antibiotic corresponding to the selectable marker. Transformants appear green and have well-established roots. If some plates are crowded with too many transformants, transplant them to a new plate in order to eliminate false-positive clones which grow due to their proximity to antibiotic-resistant clones.

6. When the transformants have grown well on the plate, transplant them into a pot with moistened soil until they mature and produce transgenic seeds. The roots of the transformants should be rinsed well with water to remove the adherent agar, which may promote fungal growth and thus damage the plants. In order to investigate the
phenotype or function of the target protein, isolation of homozygous lines from the subsequent plant generation is recommended. It is recommended that the expression of the epitope-tagged protein be confirmed by western blotting before performing immunoelectron microscopic analysis.

4. Notes

1. Chemicals used in this chapter are purchased from Sigma (St. Louis, MO) when a specific supplier is not specified. Chemicals supplied by different suppliers may be substituted.

2. Ecotype Columbia-0 (Col-0) is suitable for use in floral dipping. The ecotypes Landsberg erecta, Wassilewskija, and Nossen can also be used, although they are less efficient in terms of the rate of transformation. In cases where mutant strains (knockout lines for specific genes) are used, the corresponding parent lines should be selected as controls.

3. Mix the different types of soil well in the ratios described with the help of a scoop. This specific soil composition aids aeration and retards fungus growth. The ratios of sand and pumice or vermiculite and perlite used can be modified depending on the texture and quality of the potting soil. Fill this soil mixture into plastic pots and place the pots in a tray. Before sowing the seeds, the soil must be moistened thoroughly with water.

4. Plant growth chamber with controlled temperature, humidity, and illumination (photoperiod and intensity). For optimal growth, set the temperature of the growth chamber at 25°C. A lower temperature is acceptable, but a higher temperature can be detrimental for the growth of Arabidopsis plants. In our laboratory, the plant growth chamber is set at 23°C to ensure that the temperature does not exceed 25°C when the fluorescent lamps and heating or cooling units are switched on and off. The optimal light intensity is 80–150 μmol m⁻² s⁻¹. The light intensity can be adjusted by changing the capacity or number of daylight fluorescent lamps.

5. A variety of binary plasmids containing a pair of border sequences, a selection marker, and the origins of replication for Escherichia coli and A. tumefaciens can be obtained from the European Arabidopsis Stock Centre (http://arabidopsis.info/) and individual research groups. After choosing a binary vector, subclone the cDNA
generated by the method described above downstream of
the promoter region. Use the Cauliflower mosaic virus
35S promoter region in order to obtain constitutive high
expression. If the protein encoded by the cDNA is toxic
and could damage the host plant when expressed at a
high level, the use of an inducible promoter such as the
dexamethasone-inducible promoter instead of the 35S pro-
moter is recommended. To fuse the epitope tag sequence
to the N-terminus of the target protein, the DNA sequence
encoding the epitope should be inserted between the first
codon (ATG = Met) and the second codon in a forward-
specific primer for polymerase chain reaction (PCR). For
C-terminal labeling, insert the epitope tag sequence imme-
diately before the stop codon in a reverse-specific primer.
The DNA sequences of the different epitope tags used are
listed below.
c-Myc: GAA CAA AAA CTC ATC TCA GAA GAG GAT
FLAG: GAT TAC AAG GAT GAC GAC GAT AAG
Polyhistidine: CAT CAT CAC CAT CAC CAT CAC CAT
Repeating the epitope tag (two to three times) may
enhance the detection sensitivity, although the cloning
strategy becomes more complicated. Detection sensi-
tivity of polyhistidine is less than the others, but
polyhistidine-tagged proteins can be purified by affinity
chromatography.

6. *A. tumefaciens* transformed with the binary vector con-
struct is used as the gene carrier for transformation of the
host plant. The strain LBA4404 is commonly used for this
purpose and competent cells for electroporation are com-
mercially available from various companies. However, the
C58C1RifR and EHA101 strains exhibit higher transfor-
mation efficiency than the LBA4404 strain. If LBA4404-
mediated transformation is unsuccessful, we recommend
the use of one of these strains.

7. Plants germinated and grown under long-day conditions
can also be used. In long-day conditions, plants can be
dipped 4–6 weeks after sowing onto the soil. In this case,
sow 2–4 seeds in a pot covered with nylon window screen
secured with a rubber band to prevent the soil from falling
into the dipping bath. These plants have less rosette leaves
and thin bolts and wither easier than the plants grown
under our conditions.

8. Transformation efficiency is strongly dependent upon the
electro-pulse and gap width of the cuvette. Suitable com-
binations should be empirically determined. The condi-
tions given are optimal for transformation of *A. tumefaci-
ciens*. 
9. The concentration of *Agrobacterium* in dipping solution could be reduced to an OD$_{600}$ of 0.4 if most of the dipped plants wither due to *Agrobacterium* overgrowth.

10. To improve the transformation efficiency, vacuum infiltration (4) can be used. Because vacuum-infiltrated plants wither easily, caution should be exercised during the subsequent steps involving shade control and watering of the plants.

11. The addition of bleach may cause the formation of small air bubbles on the seed surface. These bubbles should be released by tapping or vortexing for a short period.

References


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