Video Article

Floral-dip Transformation of *Arabidopsis thaliana* to Examine *pTSO2::β-glucuronidase* Reporter Gene Expression

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Abstract

The ability to introduce foreign genes into an organism is the foundation for modern biology and biotechnology. In the model flowering plant *Arabidopsis thaliana*, the floral-dip transformation method[1-2] has replaced all previous methods because of its simplicity, efficiency, and low cost. Specifically, shoots of young flowering *Arabidopsis* plants are dipped in a solution of *Agrobacterium tumefaciens* carrying specific plasmid constructs. After dipping, the plants are returned to normal growth and yield seeds, a small percentage of which are transformed with the foreign gene and can be selected for on medium containing antibiotics. This floral-dip method significantly facilitated *Arabidopsis* research and contributed greatly to our understanding of plant gene function. In this study, we use the floral-dip method to transform a reporter gene, *β-glucuronidase (GUS)*, under the control of *TSO2* promoter. *TSO2*, coding for the Ribonucleotide Reductase (RNR) small subunit[3], is a cell cycle regulated gene essential for dNDP biosynthesis in the S-phase of the cell cycle. Examination of *GUS* expression in transgenic *Arabidopsis* seedlings shows that *TSO2* is expressed in actively dividing tissues. The reported experimental method and materials can be easily adapted not only for research but also for education at high school and college levels.

Video Link

The video component of this article can be found at https://www.jove.com/video/1952/

Protocol

I. Floral-dip transformation of *Arabidopsis thaliana*

1. Approximately 1 month prior to transformation, sow *Arabidopsis* seeds onto 4 to 8 pots (5 inch square pots) with approximately 10-15 plants per pot. For plants with normal fertility, 4 pots of plants per plasmid construct will be sufficient. If one has to transform mutant *Arabidopsis* with reduced fertility, increase the number of pots accordingly.
2. The plants are grown under standard conditions (16 hr light and 8 hr dark at 22°C). If plants are grown under shorter day or lower temperature, they will take longer to flower and longer to be ready for transformation. Good plant care will ensure healthy plants, which are essential for successful transformation.
3. When the plants have just bolted and begun to flower. They are now ready for transformation. To increase transformation efficiency, 3-4 days before the transformation, trim off the main inflorescence shoots as soon as they have bolted to encourage more secondary shoot formation. Water the plants the day before transformation. This ensures that the soil will not soak up too much infiltration media during floral-dipping.
4. Two days prior to transformation, inoculate 40ml LB containing appropriate antibiotics (in the case of *pTSO2::GUS*, 50 µg/ml Kanamycin) with *Agrobacterium tumefaciens* strain GV3101 carrying the *pTSO2::GUS* construct. The *pTSO2::GUS* is cloned in the *pBIN20* vector that possesses *NPTII* gene that confers resistance to Kanamycin[3]. Grow overnight in a shaking incubator at 28-30°C.
5. The following day, transfer 8ml of the overnight culture into 400ml of LB containing 50 µg/ml Kanamycin. Grow overnight in a shaking incubator at 28-30°C.
6. On the day of transformation, when the OD_{600} of the *Agrobacterium* culture reaches approximately 0.8 (OD_{600} between 0.5 to1 are acceptable), spin down the *Agrobacterium* overnight culture at for 8 minutes at 5000rpm.
7. Resuspend the *Agrobacterium* pellet in 1 L (liter) of infiltration media (10mM $MgCl_2$, 5% sucrose, 0.44mM 6-benzyladenine (BA), 0.3% silwet L-77, 1x Gamborg s vitamin solution and autoclaved water). The medium does not need to be autoclaved but needs to be made fresh. Pour the 1 L *Agrobacterium*-containing infiltration medium into a dish (such as 8"x15"x2" Pyrex glass dish).
8. Invert the pot (plants won't fall off) and dip all aerial parts of the plants into the infiltration solution, hold for 5 minutes one pot at a time. While all aerial parts of the plant are immersed in the infiltration medium, avoid letting soil soak up any of the infiltration media to reduce chances of fungal growth on the soil.
9. After dipping, place all dipped pots on their side on several layers of paper towel in a tray. The paper towels soak up access amount of infiltration media. Cover the tray with plastic wrap to ensure high humidity. Place the tray into plant growth chamber.

10. On the following day, remove the plastic wrap and paper towels, and place pots upright. Do not water these plants for four to five more days. Afterwards, maintain the plants normally until they set seeds and collect seeds in bulk.
11. Pour plates (150x15mm petri dish) with MS medium containing 50 µg/ml Kanamycin (weight 2.2g Murashige and Skoog basal salt without vitamin, dissolve in 500 ml water, pH to 5.8 with 1M NaOH, add 4 gram agar, autoclave, cool and add Kanamycin to 50 µg/ml final concentration). Plates are kept in 4°C until needed.
12. To select for antibiotic-resistant transgenic plants, one needs to screen 20,000 seeds at minimum. 0.1 gram is about 5000 seeds. Weight and estimate the amount of seeds.
13. Sterilize seeds by wash them in 15 ml Falcon tube with 70% ethanol for 30 seconds. Wash seeds with sterile water once. Soak the seeds for 30 seconds in a solution containing 1:10 dilution of store-bought 5% bleach (Clorox). Rinse seeds with sterile water three to six times. In sterile hood, pipette about 5000 seeds onto each MS (Kanamycin) plate, spread the seeds evenly on the plate, seal the plates with Micropore 3M tape to avoid contamination.
14. Incubate the plates at 4°C in the dark for 3-4 days, then transfer the plates to a chamber (could be the same plant growth chamber). After about 14 days, transgenic seedlings stay green but the non-transgenic ones are turning pale and dying. Transfer the transgenic seedlings to soil by slowing pulling the roots out from the medium and placed them in soil.

II. Examining *pTSO2::GUS* expression patterns

1. Harvest *pTSO2::GUS* transgenic seedlings and place them in cold 90% acetone in a glass scintillation vial or microfuge tubes that are kept on ice. Polystyrene microtiter plates (not polypropylene) can also be used if one analyzes a large numbers of samples.
2. After all samples are harvested, place the vials at room temperature for 20 minutes. During this time make up fresh staining buffer without x-Gluc (0.2% Triton x-100, 50mM $NaHPO_4$ Buffer pH7.2, 2mM Potassium Ferrocyanide, 2mM Potassium Ferricyanide) and place on ice.
3. Remove acetone from the samples and add the staining buffer.
4. Make up x- Gluc staining solution (0.2% Triton x-100, 50mM $NaHPO_4$ Buffer pH7.2, 2mM Potassium Ferrocyanide, 2mM Potassium Ferricyanide, 2mM x-gluc). Remove the staining buffer from samples and add the staining buffer containing x-Gluc to the samples.
5. Infiltrate the samples under a vacuum for 15 to 20 minutes. Release the vacuum slowly and verify that all the samples sink beneath the surface of the staining solution. If necessary, repeat infiltration until all samples sink after the vacuum is released.
6. Incubate samples at 37°C overnight in the staining buffer with x-Gluc.
7. Remove samples from incubator and remove staining buffer. Wash the samples in successive ethanol series (20%, 35% and 50% ethanol) at room temperature for 30 minutes each change.
8. Fix the tissues in FAA fixative (50% Ethanol, 5% Formaldehyde, 10% Acetic acid, rest water) for 30 minutes at room temperature. Remove FAA and add 70% ethanol. At this point the tissues can be visualized and photographed under a dissecting microscope equipped with a digital camera. A dark blue color indicates where *TSO2* is expressed (Figure 1). Alternatively, the tissues can be stored at 4°C for later viewing.

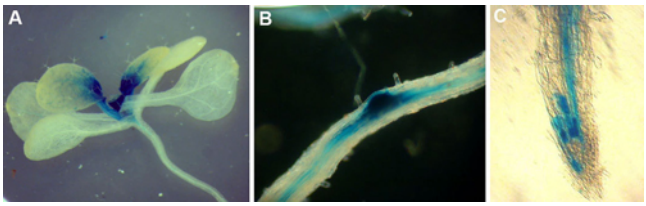

Figure 1. *pTSO2::GUS* expression in transgenic seedlings (A), lateral roots (B), and root tip (C). Note that *TSO2* promoter is highly active in young and dividing tissues. The sporadic expression pattern shown in (C) is typical of genes expressed in specific cell cycle phases.

Representative Results

When done correctly, transformation efficiency should be approximately 0.1-0.2%. In another word, one should get 5-10 transgenic seedlings by screening every 5000 seeds. On average, about 100 transgenic lines can be obtained by transforming 4 pots of wild-type plants.

For transgenic seedlings containing the *pTSO2::GUS* construct[3], dark blue color reflecting GUS activity is found in actively dividing cells including young leaves, shoot apex, root tip, and lateral root primordia (Figure 1). The non-uniform sporadic pattern is characteristic of cell cycle phase-specific expression.

Discussion

The efficiency of transformation is determined by many different factors, which are discussed below:

1. The health of the plants and their age are of primary importance. Approximately one month old plants producing numerous immature floral buds are ideal. Trimming off primary shoots to encourage secondary shoot formation 3-4 days before transformation will increase transformation efficiency. Older plants will give rise to transformants but at a lower rate.
2. Fertility of the plants is important. If one has to transform mutants with reduced fertility, a lot more plants will be needed for dipping.
3. 0.3% Silwet L-77 surfactant in infiltration buffer is considered essential.
4. Dipping time in the infiltration media should be at least 5 minutes.
5. To reduce fungal contamination, several precautions should be taken. First, one needs to avoid the soil from soaking up infiltration media by watering the plants/soil a day before infiltration. Do not submerge the soil in infiltration media. Second, remove the plastic cover and paper towel within 24 hrs after infiltration to reduce moisture. Third, do not water plants until 5 days after infiltration.

Depending on specific plasmid construct, the selection methods of transgenic plants may vary. If a plasmid vector carries the Bar (bialaphos acetyltransferase) marker gene conferring resistance to glufosinate ammonium, one can directly plant the seeds in soil without any sterilization of seeds. One then sprays the seedlings with 1:1000 diluted glufosinate ammonium (commercial name: Finale, Liberty, or Ignite) once every few days to select for resistant seedlings.

For GUS staining, it s important to harvest tissue in acetone on ice and keep all solutions cold to avoid any degradation. The staining buffers with and without X-Gluc needs to be made fresh, although the stock solution for each component can be made and stored ahead of time (see below). Potassium Ferrocyanide and Ferricyanide are toxic and should be handled with care. Additionally, it s important not to incubate the tissue at 37°C in the staining buffer with X-Gluc for longer than 1-2 days because the tissue can begin to deteriorate and/or the staining pattern may become too diffuse during the long incubations. Finally, higher ferri and ferrocyanide concentrations give lower overall staining level, but more specificity. 2mM X-Glux works well for most applications, but the concentrations may need to be adjusted for certain needs. X-Gluc is expensive. The staining should be carried out in minimal volumes.

Stock solutions that can be made ahead of time:

10% Triton X-100 (stored at room temperature for several months)
0.5 M $NaHPO_4$ Buffer (pH7.2) (stored at room temperature for several months)
100 mM Potassium Ferrocyanide (store in the dark at 4°C for several months)
100 mM Potassium Ferricyanide (Store in the dark at 4°C for several months)
100 mM X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexamine salt) is made in Dimethylformamide (DMF) and stored in -20°C in tinfoil wrapped tubes. It lasts for several months.

Disclosures

No conflicts of interest declared.

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