Chapter 8

Potato (*Solanum tuberosum* L.)

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Abstract

Agrobacterium-mediated transformation is the most common method for the incorporation of foreign genes into the genome of potato as well as many other species in the *Solanaceae* family. This chapter describes protocols for the genetic transformation of three species of potato: *Solanum tuberosum* subsp. *tuberosum* (Desiréé), *S. tuberosum* subsp. *andigenum* (Blue potato), and *S. tuberosum* subsp. *andigena* using internodal segments as explants.

Key words *Agrobacterium tumefaciens*, Andigena, β-Glucuronidase (*uidA*), Blue potato, Desiréé, Neomycin phosphotransferase II (*npt* II), Plant transformation, *Solanum tuberosum*

1 Introduction

Potato (*Solanum tuberosum* L.) is the world's third most important food crop next to rice and wheat in terms of human consumption with its production exceeding 300 million metric tons as reported by International Potato Center [1]. Potato is a critical crop in terms of food security. More than one billion population around the globe consume potato. Potato is vegetatively propagated, meaning that a whole plant can be grown from a potato tuber or a piece of it. The new plant can produce 5–20 new tubers, which will be genetic clones of the mother plant. Potato enjoys a long history of improvement through traditional breeding. Breeders target multiple traits, including resistance to biotic and abiotic stresses, and tuber quality [2].

Recently, the full sequence of the potato genome has been completed [3], opening a broad spectrum of possibilities to understand gene function and the genetic manipulation through plant transformation for the improvement of this important crop. *Agrobacterium*-mediated transformation is the most common technique used for functional genomic studies in potato, and for the introduction of novel traits into commercial potato varieties,

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while preserving combinations of desirable traits. Even though well-developed transformation protocols are available for common potato varieties, potato transformation is genotype dependent, limiting their usage and making practical applications not easily adaptable to all genotypes [4–16]. In this chapter we describe the *Agrobacterium*-mediated transformation protocols routinely used in our laboratory for three different potato cultivars, namely, Andigena, blue potato, and Desiréé. *A. tumefaciens* strain AGL1 harboring the binary vector pBI121 is used to infect the precultured internodal explants and deliver transgenes into plant cells. After cocultivation the infected internodal explants are selected on kanamycin-containing callus induction medium (CIM). Putative transgenic plants are regenerated on shoot induction medium (SIM) from selected calli. The process from explants preparation to getting a transgenic plant takes about 90 days (Fig. 1). The average transformation frequency (defined as the number of PCR-positive transgenic plants/total number of explants used) for Andigena, blue potato, and Desiréé is 35, 22, and 65 % respectively.

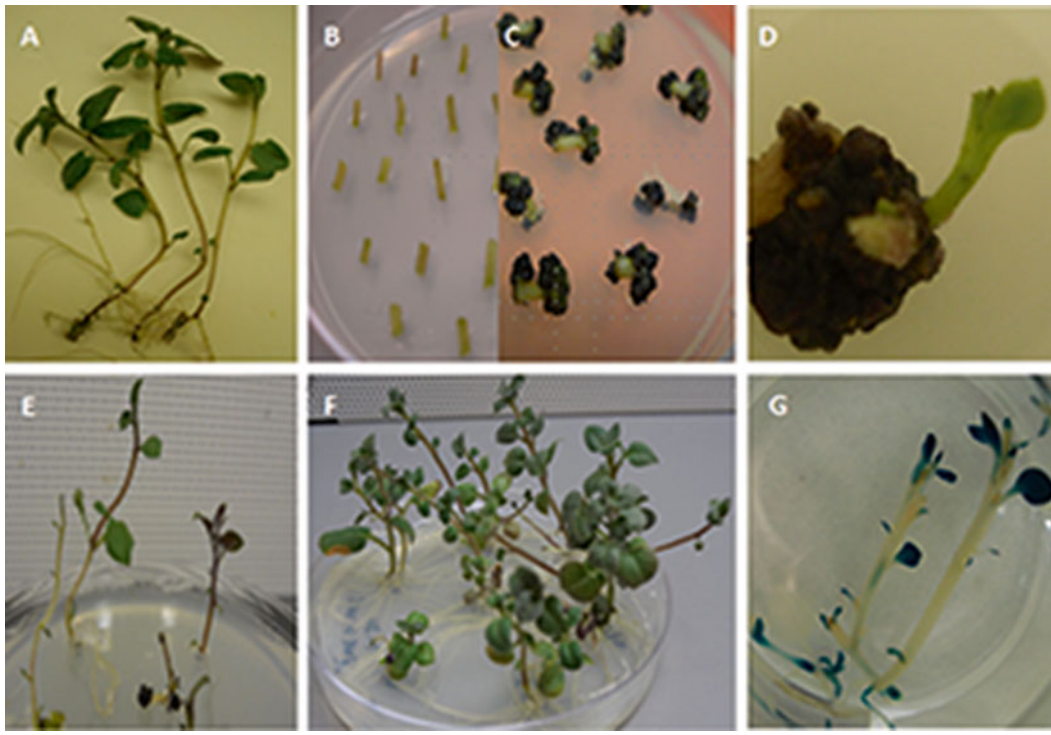

Fig. 1 Steps in the transformation of potato with *Agrobacterium tumefaciens* strain AGL1 harboring plasmid pBI121. (**a**) Four-week-old in vitro plants used as source of inter nodal explants. (**b**) Pre-culture of internodal explants on CIM for 2 days. (**c**) Callus formation on CIM with antibiotic selection in 2–3 weeks. (**d**) Shoot regeneration on SIM with antibiotic selection (4 weeks). (**e**) Shoot elongation on SIM with antibiotic selection (2 weeks). (**f**) Rooting on RIM with antibiotic selection (3 weeks). (**g**) Histochemical detection of GUS expression in transgenic shoots

2 Materials

2.1 Plant Material

In vitro micropropagated plants are used as source of stem internodal explants for transformation. *Solanum tuberosum* subsp. *tuberosum* cv Desiréé and *S. tuberosum* subsp. *andigenum* (blue potato) plants were originally established in vitro from tuber sprouts. The plants are grown at 25 °C under 16 h light/8 h dark cycle with fluorescent light (irradiance of 60 μmol/m^2/s). Four-week-old plantlets are used for the transformation studies.

2.2 Bacterial Strain and the Binary Vector

1. *Agrobacterium tumefaciens* strain AGL1.
2. Binary vector: The binary vector pBI121 (Clontech, Palo Alto, CA, USA) contains *β-glucuronidase* (*uidA*) and *neomycin phosphotransferase* II (*npt* II) genes under the regulation of the *CaMV* 35S promoter [17, 18]. The binary vector pBI121 is transformed into AGL1 using electroporation. Transformed cells are selected on YEP plates with kanamycin (100 mg/L). Kanamycin-resistant colonies are screened by colony PCR using *npt* II primers. A PCR-positive colony is grown in 5 mL of YEP with kanamycin, and *Agrobacterium* glycerol stocks are prepared by mixing equal volumes of glycerol and the *Agrobacterium* culture (1:1 ratio v/v). Glycerol stocks are stored at −80 °C.

2.3 Stock Solutions

All chemicals for stock solutions and culture media can be obtained from different vendors including Sigma-Aldrich (www.sigmaaldrich.com), Plantmedia (www.plantmedia.com), *Phyto*-Technology Laboratories (www.phytotechlab.com), and Caisson Laboratories (www.caissonlabs.com). Unless otherwise specified, all stock solutions are filter sterilized and stored at −20 °C:

1. Gamborg's B5 vitamin solution (1,000×): Dissolve 200 mg nicotinic acid, 200 mg pyridoxine hydrochloride, and 2,000 mg of thiamine hydrochloride in 180 mL of ddH_2O [19]. Bring the volume up to 200 mL with ddH_2O.
2. MS vitamin solution (1,000×): Dissolve 100 mg thiamine HCl, 50 mg pyridoxine HCl, 50 mg nicotinic acid, and 200 mg glycine in 100 mL of ddH_2O [20].
3. Indole-3 butyric acid (IBA) solution (1 mg/mL): Dissolve 20 mg of IBA in 2 mL of 95 % ethanol. Bring the volume up to 20 mL with ddH_2O.
4. Naphthalene acetic acid (NAA) solution (1 mg/mL): Dissolve 20 mg of NAA in 1 mL of 1 M NaOH. Bring the volume up to 20 mL with ddH_2O.
5. Zeatin solution (1 mg/mL): Dissolve 50 mg zeatin in 1 mL of 1 M NaOH. Bring up the volume to 50 mL with ddH_2O. Filter sterilize, dispense into1 mL aliquots, and store at −20 °C.

6. Benzyl amino purine (BAP) solution (1 mg/mL): Dissolve 50 mg BAP in a few drops of 1 N NaOH; bring the volume to 50 mL with ddH_2O. Store at 4 °C.
7. Acetosyringone solution (74 mM): Dissolve 145 mg acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone) in 10 mL of 95 % ethanol, and store at −20 °C.
8. Kanamycin sulfate solution (100 mg/mL): Dissolve 1 g of kanamycin sulfate in 5 mL ddH_2O, vortex, and bring the volume up to 10 mL with ddH_2O. Filter sterilize, dispense into 1 mL aliquots, and store at −20 °C.
9. Cefotaxime sodium salt solution (250 mg/mL): Dissolve 2.5 g of cefotaxime in 5 mL ddH_2O, vortex, and bring the volume up to 10 mL with ddH_2O. Filter sterilize, dispense into 1 mL aliquots, and store at −20 °C.
10. Carbenicillin disodium salt solution (500 mg/mL): Dissolve 5 g of carbenicillin in 5 mL ddH_2O, vortex, and bring the volume up to 10 mL with ddH_2O. Filter sterilize, dispense into 1 mL aliquots, and store at −20 °C.
11. Rifampicin solution (25 mg/mL): Dissolve 25 mg of rifampicin in 1 mL DMSO, vortex, and store at −20 °C.

2.4 Culture Media

1. YEP-*Agrobacterium* medium: 10 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl; adjust pH to 7.2 with 1 M NaOH. For solid medium, add Bacto agar (15 g/L) before autoclaving.
2. *Agrobacterium* infection medium (AIM): 4.3 g/L MS salts, 30 g/L sucrose, 1 mL MS vitamin stock solution (1,000×).
3. Clonal propagation medium (CPM): Murashige and Skoog (MS) salts [20] with sucrose 30 g/L, B5 vitamins (1×), myo-inositol 100 mg/L, naphthalene acetic acid 0.02 mg/L, agar 8 g/L, and pH 5.8.
4. Callusing, shoot regeneration, and rooting media. The composition of the medium for callus induction (callus induction medium, CIM), shoot regeneration (shoot induction medium, SIM), and rooting of shoots (root induction medium, RIM) for the three potato genotypes is presented in Table 1. The pH of all plant culture media is adjusted to pH 5.8 with 1 M KOH and sterilized by autoclaving. The hormones are added prior to autoclaving, but zeatin and all antibiotics are added after autoclaving, when the temperature of the medium has dropped to 55 °C. Sterile medium is poured into 100 × 15 mm petri dishes or magenta vessels in a laminar flow hood. All media (liquid or solid) can be stored for several weeks at 4 °C, but media with the antibiotics must be fresh.

Table 1
Composition of medium for the induction of callus, shoots, and roots from stem internode segments in three different genotypes of potato

	Desiréé			Blue potato		*S. andigena*		
Media components (L)	**CIM**	**SIM**	**RIM**	**CIM/SIM**	**RIM**	**CIM**	**SIM**	**RIM**
MS salts (g)	4.33	4.33	4.33	4.33	4.33	4.33	4.33	4.33
B5 vitamins (1,000×) (mL)	0	0	0	1	1	0	0	0
MS vitamins (1,000×) (mL)	1	1	1	0	0	1	1	1
Sucrose (g)	20	20	20	30	20	0	0	0
Glucose (g)	0	0	0	0	0	16	16	16
Myoinositol (mg)	100	100	100	100	100	100	100	100
IAA (mg)	0	0	0	0.5	0.05	0	0	0
BAP (mg)	0	0	0	0	0	0.1	0	0
NAA (mg)	0.2	0.02	0	0	0	5	0.02	0
Zeatin (mg)	2.5	2	0	1	-	0	2.2	0
GA_3 (mg)	0.02	0.02	0	0	0	0	0.15	0
pH	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8
Gelrite (g)	0	0	0	0	0	2	2	2
Agar (g)	8	8	8	8	8	0	0	0

CIM callus induction medium, *SIM* shoot induction medium, *RIM* root induction medium

2.5 Stock Solutions for DNA Isolation, PCR Reaction, and Electrophoresis [21]

1. 1 M Tris–HCl (pH 7.5): Dissolve 121.1 g Tris base in 800 mL of ddH_2O. Adjust the pH to 7.5 by adding approximately 70 mL of concentrated HCl. Adjust the volume of the solution to 1 L with ddH_2O. Store into aliquots and sterilize by autoclaving.
2. 5 M NaCl: Dissolve 146.1 g NaCl in 350 mL ddH_2O. Bring the volume up to 500 mL with ddH_2O.
3. 0.5 M ethylenediaminetetraacetic acid (EDTA, pH 8.0): Add 186.1 g of disodium EDTA to 800 mL of ddH_2O. Stir vigorously on a magnetic stirrer. Adjust the pH to 8.0 with NaOH (~20 g of NaOH pellets). Bring the volume up to 1 L with ddH_2O. Dispense into aliquots and sterilize by autoclaving (*see* **Note 1**).
4. 10 % sodium dodecyl sulfate (SDS): Dissolve 10 g SDS in 80 mL H_2O. Place in 250 mL bottle on a shaker until dissolved. Bring the volume up to 100 mL with ddH_2O. Sterilize by autoclaving.

5. DNA extraction buffer: Mix 10 mL 1 M Tris pH 7.5, 2.5 mL 5 M NaCl, 2.5 mL 0.5 M EDTA, and 2.5 mL 10 % SDS in a 50 mL Falcon tube. Bring the volume up to 50 mL with sterile ddH_2O. This buffer is stable at room temperature (RT).
6. TAE buffer (50×): Dissolve 242 g of Tris base in 700 mL ddH_2O. Add 57 mL of glacial acetic acid and 100 mL 0.5 M EDTA (pH 8.0). Bring the final volume of the mixture to 1 L with ddH_2O. Dispense in to 250 mL aliquots in 500 mL glass bottles. Sterilize by autoclaving and store at RT.
7. TE buffer: Mix 5 mL 1 M Tris–HCl pH 7.5 and 1 mL 0.5 M EDTA pH 8.0 in about 100 mL ddH_2O, and bring the volume up to 500 mL with ddH_2O. Sterilize by autoclaving. Make 25 mL aliquots and store at 4 °C.
8. Loading buffer (6×): Dissolve 25 mg of bromophenol blue in 3 mL sterile ddH_2O, add 3 mL of glycerol, and bring the volume up to 10 mL with sterile ddH_2O. Store at 4 °C.
9. Ethidium bromide: Dissolve 10 mg in 1 mL of ddH_2O. Bring the volume up to 10 mL with sterile ddH_2O. Store in a dark-brown glass bottle at RT (*see* **Note 2**).
10. 1 kb plus DNA ladder (Invitrogen): Mix 67 µL of 6× loading buffer, 100 µL of DNA ladder, and 733 µL of TE. Store at –20 °C.

2.6 Histochemical Analyses

Gus staining is a convenient way to analyze the expression of *uidA* gene, which encodes the β-glucuronidase (GUS). After staining for GUS expression, the tissue can be either examined as whole preparation or processed further to observe activity patterns in tissue sections using a microscope [17]:

1. Chloramphenicol (25 mg/mL): Dissolve 25 mg of chloramphenicol in 1 mL 100 % ethanol. Store at –20 °C.
2. Phosphate buffer pH 7.0: Dissolve 2.76 g $NaH_2PO_4 \cdot H_2O$ in 20 mL of ddH_2O. In a different beaker, dissolve 9.38 g $Na_2HPO_4 \cdot 2H_2O$ dibasic in 35 mL ddH_2O (dissolves at 37 °C). Combine 20 mL monobasic solution with 15 mL dibasic solution. Adjust the pH to 7 with dibasic solution.
3. $K_3(FeCN_6)$ 0.5 M: Dissolve 82.5 mg in 500 µL ddH_2O.
4. $K_4(FeCN_6)$ 0.5 M: Dissolve 105.5 mg in 500 µL ddH_2O.
5. Triton X-100 (10 %): Dissolve 10 g of Triton X-100 in 80 mL of ddH_2O. Bring the volume up to 100 mL and stir until well mixed. Sterilize by autoclaving and store at 4 °C.
6. X-gluc staining solution: Dissolve 100 mg of X-gluc in 200 µL *N*,*N*-dimethylformamide (DMF). Add more DMF until the solution is transparent.

7. GUS solution: Combine all the following components: 800 µL chloramphenicol (25 mg/mL), 200 µL 0.5 M $K_3(FeCN_6)$, 200 µL 0.5 M $K_4(FeCN_6)$, 4 mL 0.5 M EDTA pH 8.0, 200 µL 100 % Triton X-100, and 200 µL X-gluc solution. Bring the volume up to 200 mL with ddH_2O. Filter the solution with a 0.2 µm Millipore filter. Aliquot in 50 mL Falcon tubes (covered with aluminum foil) and store at –20 °C.

2.7 Other Solutions and Supplies

1. Sterilization solution: 20 % commercial bleach (5.25 % sodium hypochlorite) plus few drops of Tween-20.
2. Sunshine Universal Mix soil: Fosters (Waterloo, IA).
3. Sterile paper plates and towels.
4. Greenhouse standard open flat with drainage hole: McConkey, Cat # EJPFONH.
5. Clear Humi-dome 7″ (plastic, transparent): McConkey, Cat # HYFCKDOME-50. Jiffy Peat Pellets 42 mm: McConkey, Cat # JPA703.
6. Round Euro Pot 16 cm (diameter 6.25″, height 6.75″, Volume 2.5 qt/2.37 L McConkey, Cat # JMCATRI100B).

3 Methods

3.1 Growth of In Vitro Plants from Tuber Sprouts

1. Excise sprout tips (3–5 cm long) from germinating tubers under storage or tubers planted in sterile soil.
2. Rinse cut sprouts with running tap water for 5 min.
3. Place sprouts in a 50 mL sterile Falcon tube, add 50 mL of 70 % ethanol, and shake for 5 min at 50 rpm at RT.
4. Remove the ethanol and rinse sprouts once with sterile deionized distilled water.
5. Add 50 mL of sterilization solution, and shake the tube for 20 min in a shaker at 100 rpm.
6. Remove the sterilization solution and wash the sprout tips five times with sterile ddH_2O. In a laminar flow hood, place the sterilized sprouts on sterile paper towels on a paper plate to remove the excess water.
7. Sprout tips are cultured on potato clonal propagation medium.
8. Incubate the shoot tips at 25 °C under fluorescent light at 60 µmol/m^2/s with a photoperiod of 16/8 h light/dark.

3.2 In Vitro Plant Propagation Using Internodal Explants

1. Fully developed plants can be obtained from sprout tips in about 4 weeks.
2. Stem node cuttings about 5 mm long are dissected from developed plants and placed onto CPM for plant micropropagation.

3. The explants are incubated under the same conditions as the sprouts (**step 8** in Subheading 3.1).
4. Four-week-old plantlets are used as source of internodal explants for the transformation.

3.3 Preparation of Agrobacterium Culture

1. Follow standard techniques to transform *Agrobacterium* strains with a binary vector that carries the gene or genes of interest. The *Agrobacterium* strain AGL1 is stored as glycerol stocks at −80 °C.
2. To initiate the transformation take a loop full of culture from a glycerol stock of *Agrobacterium* and streak it on YEP solid medium containing the appropriate antibiotics (*see* **Note 3**). Incubate the plate at 28 °C for 2 days.
3. Inoculate a freshly grown single colony of *Agrobacterium* in 2 mL YEP with specific antibiotics. Incubate the culture in a shaker (250 rpm) for two days at 28 °C.
4. Add 100 μL of liquid *Agrobacterium* culture to 50 mL YEP with specific antibiotics, and incubate overnight at 28 °C with shaking.
5. Spin down the culture at 5,000 rpm (5,152 × *g*) for 10 min and resuspend the pellet in 10 mL of AIM.
6. Determine the OD_{600} of the *Agrobacterium* culture, and adjust the OD to 0.6 with AIM (*see* **Note 4**).
7. Add 20 μL of acetosyringone stock (74 mM) to 40 mL AIM-diluted *Agrobacterium* culture to be used for transformation.

3.4 Preparation of Explants for Agrobacterium Inoculation

1. Excise internodal explants of 5 mm length from 4-week-old propagated in vitro plants, by removing the nodal segments with a sterile sharp scalpel blade on a sterile paper plate with humid paper towels.
2. Transfer the internodal explants onto Whatman filter paper placed over CIM medium in 100 × 15 mm petri dishes.
3. Seal the plates using plastic wrap, and incubate them for 2 days in a growth room at 25 °C, under fluorescent light (60 μmol/m^2/s) with a photoperiod of 16/8 h light/dark.

3.5 Agrobacterium Infection and Cocultivation

1. Transfer the internodal explants into a Falcon tube with 40 mL of the *Agrobacterium* suspension in AIM previously prepared (*see* Subheading 3.3).
2. Incubate the explants with *Agrobacterium* cells for 20 min. Shake the tubes gently during the incubation time in a rotatory or horizontal shaker at 50 rpm.
3. Remove the *Agrobacterium* suspension and transfer the explants onto sterile paper towels. Blot dry the explants between two sterile filter papers or sterile paper towels.

This step is very critical to avoid overgrowth of *Agrobacterium* and obtain good transformation efficiencies.

4. Transfer the explants onto a new 100 × 20 mm petri dish containing a Whatman filter paper placed on CIM.
5. Seal the plates using plastic wrap, and incubate the explants for 2 days as described in **step 8** of Subheading 3.1.

3.6 Selection of Transgenic Callus and Shoot Induction

1. After 2 days of cocultivation, collect the explants in Falcon tubes and rinse them for 5 min with 40 mL of sterile ddH_2O with 250 mg/L of cefotaxime.
2. Blot dry the tissue explants between sterile filter paper and sterile paper towels and transfer them to CIM containing 500 mg/L carbenicillin, 250 mg/mL cefotaxime, and 100 mg/L kanamycin (without filter paper).
3. Seal the plates with plastic wrap and incubate them as described in **step 8** of Subheading 3.1.
4. Transfer the explants on to fresh CIM plates once every 2 weeks.
5. If *Agrobacterium* overgrowth is observed, the explants should be washed again 3× with 250 mg/L cefotaxime and 500 mg/L carbenicillin, blot dry, and continue culturing on CIM.
6. Shoot primordia start to appear after the first 4 weeks.

3.7 Shoot Elongation and Root Induction

1. Transfer explants with callus and shoot primordia to shoot induction medium (SIM) supplemented with the same antibiotics (*see* **step 2** of Subheading 3.5) in magenta boxes, and incubate as before (*see* **Note 5**).
2. After 4 weeks on SIM, transfer elongated shoots that are at least 2 cm long to test tubes containing 10 mL of RIM supplemented with 250 mg/L carbenicillin, 125 mg/L cefotaxime, 50 mg/L kanamycin, and incubate them as before.

3.8 Transplanting and Acclimation of Rooted Shoots

1. Gently remove the shoots with well-formed root systems from the test tubes. Wash off the agar medium from the roots using ddH_2O (*see* **Note 6**).
2. Transfer each in vitro plantlet to a Jiffy peat pellet and place them in a flat tray with water in the greenhouse. Use a transparent dome to cover the flat to maintain a high relative humidity for acclimation during the first 3 days.

3.9 Greenhouse Care of Transgenic Plants

1. After 1 week of acclimation, when roots are coming out of the Jiffy pellet, transfer the established potato plantlet to 2″ pots with Sunshine Universal soil mix, wet with regular water, and then move them to the greenhouse. Ensure that plants are accurately labeled. The ideal greenhouse environmental conditions for all the three potato cultivars are temperature 22 °C, relative humidity (~70 %), and natural light.

2. After 3 weeks in small pots, transfer the potato plants to 6.5″ pots with Sunshine Universal soil mix; water and fertilize them regularly using a half-strength Hoagland's solution [22].
3. Keep plants growing in the greenhouse for around 3 months for tuber production.

3.10 DNA Extraction and PCR Analysis

1. Grind 100 mg of leaves from putative transgenic plants (i.e., kanamycin resistant) in an Eppendorf tube with 450 μL of extraction buffer (200 mM Tris–HCl (pH 7.5), 250 mM NaCl, 25 mM EDTA (pH 8.0), 0.5 % SDS), followed by chloroform extraction, and isopropanol precipitation of nucleic acids.
2. Wash the DNA pellet with 70 % ethanol two times, and resuspend in 50 μL of TE buffer.
3. Dilute the DNA to a final concentration of 100 ng/μL using nuclease free water.
4. For regular end-PCR analysis, 100 ng of DNA is added to a 20 μL of PCR reaction mix.
5. PCR reaction mix contains 0.25 mM dNTPs, 2 mM $MgCl_2$, 0.5 U Ex Taq DNA polymerase (Life Technologies®, NY, USA), and 0.5 μM of each primer pair for the amplification of the *nptII* gene (forward primer: 5′-GGATTGCACGCAGGTTCTCC-3′, and reverse primer: 5′-AACTCGTCAAGAAGGCGATA-3′).
6. Reaction conditions for end-PCR are 94 °C for 5 min, followed by 29 cycles of 94 °C for 30 s, 57 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR products are visualized after electrophoresis on 0.8 % agarose gels. The gel is scored for the presence or absence of the *npt*II product (773 bp).

3.11 Histochemical Analyses for GUS Assay

1. Submerge tissue samples in GUS solution.
2. Place the tissue under vacuum for 10 min. Close the valve and let it stay for another 20 min in the dark.
3. Slowly open the valve and release the vacuum. Cover the container with Parafilm and then wrap it in aluminum foil and incubate at 37 °C for 8–16 h. Incubation time depends on the tissue and the promoter fusion being used.
4. Remove the container from the incubator, and replace the GUS solution with 50 % ethanol and incubate at 37 °C for 1 h. Repeat the process with 70–100 % ethanol until the tissue is cleared. Tissue can remain in 70–100 % ethanol indefinitely at 4 °C.
5. Look at the tissue samples under a microscope and take pictures.

4 Notes

1. The EDTA disodium salt will not go into solution until the pH of the solution is adjusted to ~8.0 by the addition of NaOH.
2. Ethidium bromide is a strong mutagen and a possible carcinogen or teratogen. Its hazardous properties require the use gloves for handling and safety disposal.
3. Rifampicin (25 mg/L) and kanamycin (50 mg/L) are added to grow *Agrobacterium* strain AGL1 transformed with pBI121.
4. To calculate the final dilution vo*lume of Agrobacterium* (V_1), use the equation $C_1V_1 = C_2V_2$, where C_1 and C_2 are respectively the initial and final OD and V_2 the final volume. Use AIM to dilute the *Agrobacterium* cells. Diluted *Agrobacterium* culture has to be used immediately to avoid aggregation.
5. Shoots regenerated from the two cut ends of the explants can be considered independent events. It is important to cut off the calli at the base from the shoot.
6. Transgenic shoots start rooting after 3–5 days.

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