Chapter 18

Initiation and Transformation of Grapevine Embryogenic Cultures

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Abstract

Protocols for the production and transformation of grapevine embryogenic cultures are described. Embryogenic cultures are initiated from leaves or stamens and pistils and transformed with *Agrobacterium* containing an enhanced green fluorescent protein/neomycin phosphotransferase II (egfp/nptII) fusion gene. Cultures are transferred to induction medium in the dark for callus formation and proliferation. Resulting cultures are transferred to somatic embryo development medium to induce secondary embryogenesis and formation of transgenic somatic embryos. Transgenic embryos identified on the basis on GFP fluorescence and kanamycin resistance are transferred to germination medium to regenerate transgenic plants. The presence of transgenes in independent plant lines is confirmed by PCR.

**Key words:** *Vitis*, Somatic embryogenesis, *Agrobacterium*, Transgenic plants, Transgenes, Culture medium, Growth regulators, Plant tissue culture

1. Introduction

Grape is a high-value crop, prized for its multiple uses as a fresh fruit and processed food product (jelly, juice, raisins, and wine). The health attributes of grape have become increasingly well documented. A host of antioxidants and vitamins found in grape and its products help prevent heart disease and cancer. These attributes have contributed to make grape the world’s most important fruit crop, including a major crop in the United States (1) where it contributes $162 billion annually to the US economy (2).

*Vitis* crop improvement is accomplished by clonal selection of spontaneous bud mutations and breeding (3, 4). However, random occurrence of bud mutations limits directed crop improvement. Breeding has limited application for genetic improvement due to extreme heterozygosity of the *Vitis* genome, which is fostered by inbreeding depression (5). Inbreeding depression makes
backcrossing and recurrent selection difficult. In particular, for varieties used in wine production where the enological characteristics are finely appreciated, it is impossible to introduce a useful trait via breeding without disrupting the desired phenotype. In addition, the long juvenile period of vines makes screening of new selections tedious and time consuming (6).

Genetic transformation involves transfer of a DNA sequence into a plant cell and its subsequent integration into the host genome. Transformation serves as an important tool in plant biology for studying gene function and expression. It also offers a potential alternative for adding single traits, such as disease resistance, to elite grapevine varieties without changing desirable characteristics (7). The essential prerequisites for a gene transfer system include the availability of a target tissue, a method to introduce foreign DNA, and a procedure to select transformed cells and regenerate transgenic plants (8).

Grapevine embryogenic cultures have been the most-used target tissue for transgene insertion and recovery of transgenic plant lines (9–14). The routine use of embryogenic cultures for transformation (7) necessitates optimization of protocols for culture initiation and maintenance. We have optimized protocols for initiation and maintenance of embryogenic cultures for several Vitis species and varieties (15, 16). Cultures can be initiated from leaves or floral explants and can be maintained on hormone-free embryo development medium for extended periods of time. Somatic embryos at the cotyledonary stage of development are used as target tissues for genetic transformation and plant regeneration.

Although biolistic bombardment has been used for delivery of transgenes into grape plant cells (17), Agrobacterium-mediated transformation is by far the most commonly used method for producing transgenic plants (18). In nature, Agrobacterium tumefaciens is a pathogenic bacterium infecting grapevine and transfers a part of its DNA (T-DNA), present on a tumor-inducing plasmid (Ti plasmid), to the nuclear genome of the host plant. Genes coding for the synthesis of amino acids known as opines are present on the T-DNA. The production of opines results in the proliferation of plant cells and formation of tumors (19). This T-DNA transfer mechanism is exploited for transformation of grapevines by replacing tumor-causing genes on the T-DNA with desired genes of interest to be studied. Such strains can no longer cause tumors upon infection and are termed “disarmed” strains. Genes of interest either can be inserted into the T-DNA region of a Ti plasmid or the T-DNA region of a smaller plasmid that is then introduced into disarmed Agrobacterium. The latter, known as a binary vector system, is most commonly adopted for transformation (20).

The success of a transformation system depends on the ability to successfully recover transformants after cocultivation. Certain genes are incorporated, along with the gene of interest, into the
T-DNA region to confirm the presence of the T-DNA in plant cells and/or confer a competitive advantage to growth of transgenic cells over nontransformed cells. These include reporter genes, which indicate the presence of the T-DNA, and selectable marker genes that most often confer antibiotic resistance to transgenic cells. The green fluorescent protein (GFP) gene isolated from the Pacific jellyfish *Aequorea victoria* is routinely used as a reporter gene in grapevine transformation studies (10, 11). Plant cells expressing GFP produce a bright green fluorescence, which can be observed under a stereomicroscope equipped for epifluorescence illumination. Alternatively, GFP can be visualized in plant cells using a relatively inexpensive detection system (21). Selectable marker genes such as neomycin phosphotransferase II (*npt II*) and hygromycin phosphotransferase (*hpt*) genes are frequently used along with reporter genes in genetic transformation (22, 23). Transgenic cells carrying these marker genes can selectively grow on the culture medium containing kanamycin or hygromycin antibiotics, while inhibiting the growth of nontransformed cells.

This chapter presents methods for the production and transformation of embryogenic cultures to regenerate transgenic plants with desired genes of interest. Embryogenic cultures initiated either from leaves or floral explants are cocultivated with *Agrobacterium* containing a binary plasmid with the genes of interest. Transformed somatic embryo lines are identified based on GFP fluorescence and kanamycin resistance. Regenerated plants obtained from germinated transgenic embryos are hardened in a growth room and transferred to a greenhouse. Presence of transgenes is estimated using polymerase chain reaction (PCR).

### 2. Materials

#### 2.1. Supplies and Equipment

1. Laminar flow hood.
2. 100-mm × 15-mm plastic Petri dishes.
3. 50-mL centrifuge tube.
4. 125-mL conical flasks.
5. GA-7 Magenta vessel.
6. Scalpels.
7. Forceps.
8. Sterile Whatman 3MM filter paper.
11. Clorox®.
13. Micropipettors and micropipette tips.
14. 70% ethanol.
15. Tween 20.
16. 96-well PTC-100 programmable thermal controller (MJ Research, Watertown, MA).
17. 500-µL centrifuge tubes.
18. Growth chamber.
19. Leica MZFLIII stereomicroscope equipped for epifluorescence with an HBO 100-W mercury lamp illuminator and a GFP filter set composed of an excitation filter (470/40 nm), a dichromatic beam splitter (485 nm), and a barrier filter (525/50 nm) (Leica Microscopy System Ltd., Heerbrugg, Switzerland).
20. Sunrise optical microplate reader (Phenix Research Products, CA, USA).

### 2.2. Plant Tissue Culture Media

1. Embryogenic culture induction medium from leaves (NB2 medium), pH 6.0: (28) salts and vitamins, 20 g/L sucrose, 0.1 g/L myo-inositol 1.0 µM BAP, 5.0 µM 2,4-D, and 7.0 g/L TC agar (see Note 1).
2. Embryogenic culture induction medium from floral explants (MSI medium), pH 6.0: (27) salts and vitamins, 20 g/L sucrose, 0.1 g/L myo-inositol, 4.5 µM BAP, 5.0 µM 2,4-D, and 7 g/L TC agar (see Note 2).
3. Embryogenic culture induction medium from floral explants (PIV medium), pH 5.7: (28) salts, B5 vitamins, 60 g/L sucrose, 8.9 µM BAP, 4.5 µM 2,4-D, and 3.0 g/L Phytagel (see Note 3).
4. Somatic embryo (SE) development and maintenance medium (×6 medium), pH 5.8: MS medium lacking glycine and supplemented with 3.033 g/L KNO₃ and 0.364 g/L NH₄Cl (as the sole nitrogen source), 60.0 g/L sucrose, 1.0 g/L myo-inositol, 7.0 g/L TC agar, and 0.5 g/L activated charcoal (see Note 4).
5. Liquid cocultivation medium (DM medium), pH 5.7: DKW basal salts (26), 0.3 g/L KNO₃, 1.0 g/L myo-inositol, 2.0 mg/L each of thiamine-HCl and glycine, 1.0 mg/L nicotinic acid, 30 g/L sucrose, 5.0 µM BA, and 2.5 µM each NOA and 2,4-D.
6. Callus induction medium (DM medium), pH 5.7: DKW basal salts (26), 0.3 g/L KNO₃, 1.0 g/L myo-inositol, 2.0 mg/L each of thiamine-HCl and glycine, 1.0 mg/L nicotinic acid, 30 g/L sucrose, 5.0 µM BA, 2.5 µM each NOA, 2,4-D,
7.0 g/L TC agar, 200 mg/L each of carbenicillin and cefotaxime, and 100 mg/L kanamycin.

7. Somatic embryo germination medium (MS1B), pH 5.8: MS salts and vitamins, 20.0 g/L sucrose, 0.1 g/L myo-inositol, 1.0 μM BAP, and 7.0 g/L TC agar.

8. Solid *Agrobacterium* culture medium: yeast extract peptone (YEP) medium (10 g/L peptone, 10 g/L yeast extract, 5 g/L NaCl, pH 7.0) supplemented with 15 g/L Bacto agar.

9. Liquid MG/L medium, pH 7.0: 5.0 g/L mannitol, 1.0 g/L l-glutamate, – 5.0 g/L tryptone, 2.5 g/L yeast extract, 5.0 g/L NaCl, 150.0 mg/L KH₂PO₄, 100.0 mg/L MgSO₄.7H₂O, and 2.5 mL/L Fe-EDTA (To make a stock solution of Fe-EDTA, dissolve 7.44 g of Na₂EDTA·2H₂O and 1.86 g FeSO₄.7H₂O in sterile distilled water and make final volume to 1 L.) (see Note 5).

10. Liquid 2× medium, pH 5.8: x6 medium modified to contain 20.0 g/L sucrose without TC agar and activated charcoal.

### 2.3. In Vitro Plant Materials

1. In vitro micropropagation cultures (see Note 6).

2. Dormant vine cuttings (see Note 7).

### 2.4. Antibiotic Stock Solutions

1. Rifampicin: 20 mg of antibiotic dissolved in 500 μL of methanol (see Note 8).

2. Kanamycin sulfate: 100 mg of antibiotic dissolved in 1 mL of water and filter-sterilized using a 0.2-μm membrane.

3. Carbenicillin and cefotaxime: 200 mg of each antibiotic dissolved in 1 mL of water and filter-sterilized using a 0.2-μm membrane.

### 2.5. Agrobacterium Culture

1. Binary vector containing an *egfp/nptII* fusion gene under the control of a double cassava vein mosaic virus (CsVMV) promoter.

2. *Agrobacterium* culture stock containing the binary vector (stored in glycerol at −70°C).

### 2.6. PCR Reagents

1. *Taq* DNA polymerase.

2. 10× PCR buffer with Mg²⁺.

3. dNTP mixture.

4. Primers

*egfp*-specific oligonucleotide primer pairs EG-51 (5′-ATG GTG AGC AAG GGC GAG GAG CTG T-3′) and EG-32 (5′-CTT GTA CAG CTC GTC CAT GCC GAG A-3′).
3. Methods

3.1. Initiation of Embryogenic Cultures from Leaves

1. Excise 1.5–5.0-mm-long unopened leaves from in vitro micropropagation cultures and place five leaves on a Petri dish containing NB2 medium (see Note 9).

2. Cover Petri dishes in aluminum foil and incubate in darkness at 26°C for 5–7 weeks.

3. Transfer Petri dishes containing the callus cultures to cool white fluorescent light (65 μmol/m²/s and 16-h photoperiod) at 26°C for 5 weeks. Screen callus cultures at weekly intervals.

4. Explants will produce sectors of compact, cream-colored embryogenic callus and loose, brown-colored nonembryogenic callus (see Note 10).

5. Transfer embryogenic callus to growth regulator free ×6 medium for the development and proliferation of proembryonic masses (PEM) and somatic embryos (SE).

6. Maintain embryogenic cultures by transferring gray-colored PEM to fresh ×6 medium at 4–6 week intervals.

7. Use cotyledonary stage SE for Agrobacterium-mediated transformation.

3.2. Initiation of Embryogenic Cultures from Floral Explants

1. Obtain inflorescences from either field grown grapevines or dormant vine cuttings (see Note 11).

2. Determine developmental stages of stamens and pistils and select the optimum stage that produces an embryogenic response (see Note 12).

3. Immerse inflorescences in 70% ethanol for 30 s followed by washing them in distilled water for 30 s.

4. Wash inflorescences in 25% NaOCl solution containing one drop Triton X-100 with constant agitation for 5 min, followed by three, 5-min washes in sterile distilled water.

5. Carefully separate stamens (anthers with intact filaments) from the calyptra and the pistil before placing them on MSI or PIV medium. Also, place the pistil with the remaining filament stubs on the medium. Place 35 stamens in a clump in the center of a Petri dish and five pistils near the perimeter.

6. Seal Petri dishes with Parafilm® and place in darkness at 26°C for 4 weeks.

7. After 4 weeks, transfer Petri dishes under cool white fluorescent lights (65 μmol/m²/s and 16-h photoperiod). Screen developing cultures using a microscope for the presence of embryogenic callus at weekly intervals for 16 weeks.
8. Production of embryogenic callus will be observed from the filament tip or connective tissue of anthers and in some cases from pistils (15).

9. Transfer embryogenic callus to ×6 medium for development and proliferation of SE. Maintain embryogenic cultures by transferring gray-colored PEM to fresh ×6 medium at 4–6 week intervals (see Note 13).

### 3.3. Initiation of Agrobacterium Culture

1. Transfer *Agrobacterium* culture containing the binary plasmid from −80°C to room temperature and thaw.

2. Spread approximately 20 μL of bacterial culture on a Petri dish containing solid YEP medium with 20 mg/L rifampicin and 100 mg/L kanamycin. Incubate dishes in the dark at 26°C for 3 days.

3. Isolate a single colony growing on YEP medium and transfer it to a 125-mL conical flask containing 30 mL MG/L medium with 20 mg/L rifampicin and 100 mg/L kanamycin.

4. Incubate on a rotary shaker at 180 rpm at 26°C for 16–20 h. The bacterial culture should appear cloudy at the end of the culture period.

5. Transfer the culture to a 50-mL centrifuge tube and spin at 2,220×g for 8 min at room temperature. Discard the supernatant and resuspend the pellet in 30 mL liquid 2× medium. Adjust OD value to 0.6 using liquid 2× medium.

6. Transfer the contents of the tube to a 125-mL conical flask and incubate for an additional 4 h on a rotary shaker under the same conditions as above. Use this culture for cocultivation.

### 3.4. Transformation of Somatic Embryos

1. Transfer cotyledonary stage SE to sterile Petri dishes. Avoid wounding SE during transfer to prevent browning (see Note 14).

2. Add 5.0 mL *Agrobacterium* culture to the SE and mix thoroughly by swirling. Incubate for 7 min. Blot SE dry on filter paper to remove the bacteria.

3. Transfer blotted SE to a Petri dish containing two layers of filter paper soaked in liquid DM medium (see Note 15).

4. Seal the Petri dish with Parafilm® and cocultivate in darkness at 26°C for 3 days.

5. After 3 days, observe SE for transient GFP expression using a Leica MZFLIII stereomicroscope equipped for epifluorescence.

6. Transfer cocultivated SE to a 125-mL conical flask containing liquid DM medium with 200 mg/L each of carbenicillin and cefotaxime and 15 mg/L kanamycin.

7. Transfer the flask to a rotary shaker at 110 rpm and wash SE for 3 days to eliminate bacterial cells.
8. Transfer washed SE to each 100-mm × 15-mm Petri dish containing 25 mL solid DM medium with 200 mg/L each of carbenicillin and cefotaxime and 100 mg/L kanamycin.

9. Place Petri dishes in dark at 26°C for 4 weeks to permit callus development and proliferation.

10. After 4 weeks, transfer callus cultures to 100-mm × 15-mm Petri dishes containing 30 mL ×6 medium with 200 mg/L each of carbenicillin and cefotaxime and 70 mg/L kanamycin for secondary embryo development. Place Petri dishes in dark and screen at weekly intervals for the presence of transgenic SE lines.

11. Independent SE lines can be identified by bright GFP fluorescence and can be used to separate transgenic lines from nontransformed SE.

12. Designate each transgenic SE line as an independent event and transfer to fresh ×6 medium with 200 mg/L each of carbenicillin and cefotaxime and 70 mg/L kanamycin.

3.5. Germination of SE Lines and Plant Regeneration

1. Transfer five SE at the late cotyledonary stage from each independent transgenic line to 100-mm × 15-mm Petri dishes containing 25 mL MS1B medium. Place Petri dishes under cool white fluorescent lights at 65 μmol/m²/s and 16-h photoperiod.

2. After 2 weeks of culture, when SE become enlarged and pigmented, excise the enlarged cotyledons to enhance subsequent shoot development (see Note 16).

3. Transfer resulting plants having a robust root and shoot system to Magenta GA-7 vessels containing 30 mL MS medium. Place vessels under cool white fluorescent lights at 65 μmol/m²/s and 16-h photoperiod for further of transgenic plant lines and a nontransformed plant using CTAB development.

4. Transfer plants to 7-cm plastic pots containing Pro-Mix BX potting mix (Premier Horticulture Inc., Red Hill, PA) and acclimate in a growth room for 2 weeks before transfer to a greenhouse (see Note 17).

3.6. PCR Analysis of Transgenic Plants

1. Isolate genomic DNA from leaves using CTAB protocol (24).

2. Make a 10 μL reaction mixture containing 1.0 μL each of the forward and reverse primer, 1.0 μL genomic DNA (concentration 20 ng/μL), 1.0 μL 10x buffer, 0.6 μL MgCl₂, 1.6 μL dNTP (concentration 1.25 mM), 0.1 μL Taq polymerase, and 3.7 μL water.

3. Conduct PCR reactions with one cycle at 95°C for 4 min, 39 cycles at 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, and a final cycle at 72°C for 4 min.
4. Use plasmid DNA as a positive control and DNA from nontransformed plants as a negative control.

5. Load PCR products on a 0.6% agarose gel containing 1.0 μL of ethidium bromide (1.0 mg/mL). Run the PCR products at 100-V current for 30 min in a gel electrophoresis unit and observe separated DNA bands with a UV transilluminator.

4. Notes

1. Recipes for media are per liter final volume. Media are autoclaved at 121°C and 15 psi pressure for 20 min. pH was adjusted using KOH prior to autoclaving.

2. Embryogenic response from leaves and floral explants is genotype dependent. In general, a greater number of varieties produce embryogenic cultures from floral explants than leaves. This factor needs to be considered prior to culture initiation from a specific explant.

3. Production of embryogenic response on MSI and PIV medium is genotype dependent and needs to be standardized for the genotype being studied.

4. An important factor affecting maintenance of embryogenic cultures is the use of TC agar as a gelling agent. Use of other gelling agents including Bacto agar and Phytagel in ×6 medium results in a rapid decline in embryogenic competence and eventual termination of cultures.

5. Although several bacterial media have been used for culture of Agrobacterium, MG/L tends to yield better cell growth and a consistent culture quality. Therefore, we recommend this medium for routine use in transformation of grapevine.

6. In vitro micropropagation cultures can be established from shoot tips of rapidly growing field grapevines on C2D4B medium following the protocol established by Gray and Benton (25). Once established, unopened leaf explants can be obtained from these cultures.

7. Dormant vine cuttings can be obtained after pruning of grapevines during the winter season. Alternatively, cuttings can be obtained from the Foundation Plant Services, University of California, Davis, CA.

8. Antibiotic stock solutions are filter-sterilized and stored at −20°C. Solutions are thawed just prior to use. Antibiotics are added to culture medium after autoclaving and cooling the medium to 55°C. Rifampicin is light sensitive and should be stored and used away from light.
9. We use *V. vinifera* “Thompson Seedless” in the majority of our experiments because of its high embryogenic potential from leaves and ability to recover a large number of independent transgenic plant lines (up to 150 lines from 1.0 g of embryogenic culture) after transformation.

10. A positive embryogenic response can be obtained only from unopened leaves, 1.5–5.0 mm in size. Larger size leaves will produce a nonembryogenic callus and should not be used to initiate cultures.

11. Dormant vine cuttings can be forced to flower by placing them in 500-mL Erlenmeyer flasks containing 250 mL sterile distilled water under cool white fluorescent lights (65 μmol/m²/s and 16-h photoperiod), at 26°C for 3 weeks. Replace the water and trim the basal 1 cm of each cutting at weekly intervals to avoid fungal contamination.

12. The developmental stage of stamens and pistils suitable for obtaining an embryogenic response is variety dependent. In general, stage II and stage III stamens and pistils (15) produce the best embryogenic response among a large number of varieties.

13. It is critical to transfer embryogenic cultures to fresh ×6 medium at 4–6 week intervals. Failure to do so will result in a decrease in regeneration potential of SE and subsequent reduction in transformation efficiency and plant regeneration.

14. Grapevine SE proliferates by direct secondary embryogenesis with new embryos emerging from epidermal or subepidermal cells of primary SE. Thus, surface cells of cotyledonary stage SE are the best target tissues for *Agrobacterium*-mediated transformation.

15. Cocultivation of SE on filter paper is beneficial as it reduces over growth of bacterial cells resulting in a dramatic decrease in cell necrosis and higher transformation efficiency.

16. Cotyledon excision to improve shoot recovery from germinated SE is variety dependent and needs to be tested for each variety being studied.

17. Transformation efficiency varies among *Vitis* species and varieties (18). The highest recovery of transgenic plant lines has been recorded in *V. vinifera* “Thompson Seedless.”
References