METHODS IN MOLECULAR BIOLOGY™ 344

Agrobacterium Protocols

Second Edition

Volume 2

Edited by

Kan Wang

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Edited by

Kan Wang

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and Department of Agronomy,
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To Marc Van Montagu and Jeff Schell (1935–2003), my PhD mentors, for their inspiration and encouragement.

Preface

Agrobacterium tumefaciens is a soil bacterium that for more than a century has been known as a pathogen causing the plant crown gall disease. Unlike many other pathogens, *Agrobacterium* has the ability to deliver DNA to plant cells and permanently alter the plant genome. The discovery of this unique feature 30 years ago has provided plant scientists with a powerful tool to genetically transform plants for both basic research purposes and for agricultural development.

Compared to physical transformation methods such as particle bombardment or electroporation, *Agrobacterium*-mediated DNA delivery has a number of advantages. One of the features is its propensity to generate single or a low copy number of integrated transgenes with defined ends. Integration of a single transgene copy into the plant genome is less likely to trigger "gene silencing" often associated with multiple gene insertions.

When the first edition of Agrobacterium *Protocols* was published in 1995, only a handful of plants could be routinely transformed using *Agrobacterium*. *Agrobacterium*-mediated transformation is now commonly used to introduce DNA into many plant species, including monocotyledon crop species that were previously considered non-hosts for *Agrobacterium*. Most remarkable are recent developments indicating that *Agrobacterium* can also be used to deliver DNA to non-plant species including bacteria, fungi, and even mammalian cells.

While the list of organisms that can be infected by *Agrobacterium* has increased significantly over the past decade, the success in transformation also relies on culture responsiveness of the target cells/tissues subsequent to the cocultivation with *Agrobacterium*. Essentially, the dynamic interactions between the two living organisms are critical for development of transformation methods.

The second edition of Agrobacterium *Protocols* contains 80 chapters (two volumes) divided into 14 parts. In addition to basic *Agrobacterium* handing techniques and model plant transformation methods (Parts I and II in Volume 1), this edition collects 61 chapters covering protocols for 59 plant species. Volume 2 contains 35 of 59 plant protocols (Parts I to VII). The plants are grouped according to their practical utilization rather than their botanical classification. The significant expansion of this section reflects the remarkable advancements in plant transformation technology during the past decade. Part

VIII (Non-Plants) contains six chapters with protocols for introducing DNA into non-plant species such as bacteria, fungi, algae, and mammalian cells. The description of this unique capacity of *Agrobacterium* is a new addition to this edition.

Agrobacterium *Protocols* provides a bench-top manual for tested protocols involving *Agrobacterium*-mediated transformation. All chapters are written in the same format as that used in the Methods in Molecular Biology series. Each chapter is contributed by authors who are leaders or veterans in the respective areas. The Abstract and Introduction sections provide outlines of protocols, the rationale for selection of particular target tissues, and overall transformation efficiency. The Materials section lists the host materials, *Agrobacterium* strains and vectors, stock solutions, media, and other supplies necessary for carrying out these transformation experiments. The Methods section is the core of each chapter. It provides a detailed step-by-step description of the entire transformation procedure from the preparation of starting materials to the harvest of transgenic plants. To ensure the reproducibility of each protocol, the Notes section supplies additional information on possible pitfalls in the protocol and alternative materials or methods for generating transgenic plants.

Typically, most laboratories only work on one or a few plant species. Of course, each laboratory or individual researcher has his/her own favorite variation or modification of any given plant transformation protocol. The protocols presented in this edition represent the most efficient methods used in the laboratories of these contributors. They are by no means the only methods for successful transformation of your plant of interest. The broad range of target tissue selection and in vitro culture procedures indicate the complexity in plant transformation. It is the intention of this book to facilitate the transfer of this rapidly developing technology to all researchers for use in both fundamental and applied biology. I take this opportunity to thank all my colleagues whose time and effort made this edition possible. Special thanks go to my family for their unconditional love and support during the process of editing this book.

Kan Wang

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Root Plants

1

Carrot (*Daucus carota* L.)

Owen Wally, Jayaraj Jayaraman, and Zamir K. Punja

Summary

Plants are susceptible to infection by a broad range of fungal pathogens. Many horticulturally important crop species lack adequate genetic resistance to disease. Studies on potential mechanisms of disease resistance in plants have revealed the importance of a range of pathogenesis-related (PR) proteins with antifungal activity in reducing colonization of plant tissues by pathogens. We are evaluating a range of PR-proteins, through heterologous expression in transgenic carrot tissues, for their effects on fungal disease development. The protocols for carrot transformation with a thaumatin-like protein are described. In addition, the use of herbicide resistance as a selectable marker in carrot transformation is illustrated. In this protocol, petiole segments from carrot seedlings are exposed to *Agrobacterium* for 10–30 min and co-cultivated for 3 d, after which herbicide selection is imposed until embryogenic calli are produced after 8–12 wk. The transfer of the embryogenic calli to hormone-free medium yields transgenic plantlets. This genetic transformation protocol has supported the generation of transgenic carrot plants with defined T-DNA inserts at the rate of between 1 and 3 Southern positive independent events out of 100.

Key Words: Antifungal proteins; disease resistance; genetic engineering; pathogenesis-related (PR) proteins; herbicide resistance.

1. Introduction

Carrot (*Daucus carota* L. subsp. sativus), a member of the family *Apiacaeae*, is grown worldwide for its edible taproot, which provides a source of vitamin A and fiber in the diet. One of the greatest challenges to carrot production is the management of fungal diseases. There are a number of widespread pathogens that destroy the foliage and roots of *P. carota*, thereby reducing quality and yield. Genetic resistance to fungal pathogens is lacking in most commercially grown carrot cultivars in use today.

The utility of genetic engineering approaches to enhance resistance of plants to a range of fungal pathogens has been reviewed ***(1–3)***, and can provide an opportunity

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to enhance disease resistance in carrots. Our current research involves the expression of a number of pathogenesis-related (PR) proteins with anti-fungal activity (chitinases, thaumatin-like proteins [TLP], peroxidases and β-1, 3 glucanases) in carrot tissues and the assessment of their effects on fungal pathogen development in vivo. We describe the protocols for engineering carrot plants to express TLP and the use of the *bar* gene, encoding for phosphinothricin acetlytransferase for herbicide resistance, as a selectable marker *(4)*. The methods used for assessing transgenic plants for resistance to fungal pathogens are described elsewhere *(5)*.

2. Materials

2.1. Media and Hormone Solutions

1. Murashige and Skoog (MS) medium *(6)* (containing 332.16 mg/L $CaCl_2$, 0.025 mg/L $CoCl_2 \cdot 6H_2O$, 0.025 mg/L $CuSO_4 \cdot 5H_2O$, 27.8 mg/L $FeSO_4 \cdot 7H_2O$, 6.2 mg/L H_3BO_3, 170 mg/L KH_2PO_4, 0.83 mg/L KI, 1900 mg/L KNO_3, 370 mg/L $MgSO_4 \cdot 7H_2O$, 16.9 mg/L $MnSO_4 \cdot H_2O$, 37.3 mg/L Na_2 · ethylene diamine tetraacetic acid (EDTA), 0.25 mg/L $Na_2MoO_4 \cdot 2H_2O$, 1650 mg/L NH_4NO_3 and 8.6 mg/L $ZnSO_4 \cdot 7H_2O$. Supplemented with 2 mg/L glycine, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine-HCl, 0.1 mg/L thiamine-HCl.), full and half- strength, in liquid and solid form, supplemented with various concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.3% w/v sucrose. MS1D is supplemented with 1 mg/L 2,4-D, MS(1/2)D is supplemented with 0.5 mg/L 2,4-D and MS($^1/_4$) D is supplemented with 0.25 mg/L 2,4-D. Media are used for tissue culture, plant transformation, and plantlet regeneration. The pH is adjusted to 5.8 with 1*M* KOH; phytagel (2.5 g/L) is added prior to autoclaving (*see* **Notes 1–4**).
2. Yeast extract-mannitol (YM) medium (for growth of *Agrobacterium tumefaciens*): 0.04% yeast extract, 1% mannitol, 1.7 m*M* NaCl, 0.8 m*M* $MgSO_4$ and 2.2 m*M* K_2HPO_4. The pH is adjusted to 7.2 with 1*M* NaOH prior to autoclaving. Used as a liquid or agar medium (15 g/L Bacto-agar).
3. 2,4-D stock solution (0.5 mg/mL): 50 mg of 2,4-D dissolved in 5 mL ethanol and gradually diluted to 100 mL with H_2O and stored at 4°C in dark for up to 3 mo. 2,4-D is added to media prior to autoclaving.
4. Acetosyringone stock solution (0.1 *M*): 1 g acetosyringone is dissolved into 50 mL dimethyl sulfoxide (DMSO) and stored at 4°C in foil-wrapped containers. It crystallizes at this temperature but will turn to liquid again once returned to room temperature; 2 μL of this stock added to 1 mL of bacterial medium or 2 mL stock to 1 L of co-cultivation medium would give a final concentration of 200 μ*M*.
5. Antibiotic stock solutions: Kanamycin sulphate (Sigma) (100 mg/mL), streptomycin sulfate (Sigma) (200 mg/mL) and timentin (30:1 ticarcillin: clavulanic acid 300 mg/mL, SmithKline Beecham). All antibiotics are dissolved in H_2O and filter-sterilized through 0.22 μm filters, and stored at –20°C.
6. DL- phosphinothricin stock solution: 250 mg of DL- phosphinothricin (RPI Research Products International Corp. IL) is dissolved in 25 mL of H_2O, filter-sterilized through 0.22 μm filters, and stored at –20°C.

7. Liberty Solution: The herbicide Liberty (Aventis, Saskatoon, Saskatchewan) is diluted in H_2O to between 0.2 and 0.4% (w/v) and used as an aerosol or painted on the leaf surface using a cotton swab.
8. Potting medium: Soil mix 4 (Sunshine, Surrey, British Columbia), containing 55–60% Canadian Sphagnum peat moss, perlite, dolomitic limestone (for pH adjustment) and gypsum.

2.2. Carrot Cultivars

1. Open-pollinated carrot cultivars evaluated: Nantes Coreless, Danvers Half-long, Nanco, Golden State, Scarlet Nantes, and the experimental high β-carotene producing HCM line. HCM seeds were donated by Dr. Phil Simon (USDA, University of Wisconsin, Madison, WI), while the other lines were purchased at a local wholesale seed supplier.

2.3. Agrobacterium tumefaciens *Strain and Binary Vector*

1. *Agrobacterium tumefaciens* LBA4404 Electromax cells (Invitrogen): The strain contains the disarmed Ti plasmid pAL4404 which contains only the *vir* and ori region of the Ti plasmid.
2. Binary vector pCambia-bar-TLP: The construct contains two selectable marker genes, *bar* and *hpt*, driven by the CaMV 35S promoter, and a rice *tlp* gene (1 kb) (*7*) driven by the maize ubiquiton promoter (2 kb). The ubiquitin-tlp fragment (3 kb) can be released through digestion of the entire vector with *Hin*dIII.

2.4. DNA Extraction Specifically for Carrot Tissue

Mature carrots may have high levels of phenolic and other compounds that exist at sufficient levels to inhibit polymerase chain reaction (PCR) amplifications of the deoxyribonucleic acid (DNA) samples. Therefore, specialized DNA extraction techniques have been developed to minimize the levels of potential inhibitors while still maintaining high yield and quality of genomic DNA.

1. Extraction buffer: 2 % (w/v) cetyltrimethylammonium bromide (CTAB), 1.4 *M* NaCl, 20 m*M* EDTA, 100 m*M* Tris-HCl and 5 % (w/v) polyvinylpyrrolidone (PVPP), pH 8.0, stored at room temperature.
2. Phenol:chloroform:isoamyl alcohol: buffer saturated phenol, pH 8.0 (Fisher), is mixed with 0.1% (w/v) hydroxyquinoline and mixed with an equal volume of chloroform: isoamylalcohol (24:1), stored away from light at 4°C.
3. TE buffer: 10 m*M* Tris-HCl and 1 m*M* (EDTA), pH 8.0, autoclaved at 15 psi for 15 min.

2.5. Polymerase Chain Reaction

1. 10X buffer: 200 m*M* Tris-HCl pH 8.4; 500 m*M* KCl.
2. 50 m*M* $MgCl_2$ (Invitrogen).
3. 10 m*M* dNTPs (equal volumes of individual 100 m*M* dNTP [Invitrogen] stocks are mixed and diluted 10-fold with H_2O. Diluted stocks are aliquoted into small volumes and stored a –20°C).

4. Primers for amplifying the TLP gene sequence:
   a. Forward primer: tlpF 5′-AACAGGTGCCAGTACACGGTGT-3′.
   b. Reverse primer: tlpR 5′-CACGGTTACATCCACACATGCA-3′.
5. Primers for amplifying the bar gene sequence:
   a. Forward primer: BarF 5′-ACTGGGCTCCACGCTCTAC-3′.
   b. Reverse primer: BarR 5′- GAAGTCCAGCTGCCAGAAAC-3′.
6. Recombinant Taq-DNA polymerase (5 U/μL, Invitrogen).
7. The PCR machine used was a GeneAmp PCR system 9700 (Perkin-Elmer Corporation, Norwalk, CT).

3. Methods

3.1. Sterile Carrot Tissue

1. Seeds should be soaked overnight in H_2O at 4°C, and then washed twice with H_2O and once for 5 min with 70% ethanol. The seeds are then soaked in 1% (v/v) NaOCl with a drop of Tween-20 for 15 mins and washed four times with sterile H_2O.
2. Sterilized seeds are blotted dry on sterile filter paper and approx 10–15 seeds are placed in Magenta boxes containing 50 mL of half-strength MS medium supplemented with 0.3% sucrose and incubated for 4–6 wk at room temperature (22°C) under cool white fluorescent lights (450 μmol/m^2/s, 16 h d). Once the plants reach a size of 15–20 cm (6 wk) they are used as source material for transformations.

3.2. Agrobacterium *Cultures*

1. LBA 4404 electrocompetent cells are thawed on ice and 20 μL added to 100 ng of pCambia-bar-TLP binary vector *(7)* and mixed by swirling with the pipett tip. The mixture is added to a 0.1 cm cuvett and electroporated at 2 kV, 200 Ω, and 25 μF.
2. Following electroporation, 1 mL of YM broth is added, and the mixture removed and transferred to a round-bottomed 15-mL Falcon tube.
3. The contents are agitated on a rotary shaker at 225 rpm and 30°C for 3 h, the cells are then diluted at a 1:10 ratio with fresh YM and plated on solid YM medium containing 100 mg/L streptomycin and 50 mg/L kanamycin.
4. Plates are incubated for 56 h at 30°C. Single colonies are isolated and inoculated to fresh YM media.
5. The presence of the binary vector in *Agrobacterium* is confirmed by plasmid minipreparation of *Agrobacterium* followed by restriction enzyme digestion and gel electrophoresis.
6. For carrot transformation, a single colony of LBA4404 containing the plasmid pCambia-bar/TLP is used to inoculate 50 mL of liquid YM supplemented with 100 mg/L streptomycin and 50 mg/L kanamycin, and is grown overnight on a rotary shaker (250 rpm) at 30°C.
7. Cultures are centrifuged at 3000*g* at room temperature for 15 min. The supernatant is removed and the pellet is resuspended to a density of $OD_{600} = 0.3$ (corresponding to approximately 1×10^8 cells/mL) in 1/10th MS media supplemented with 200 μ*M* acetosyringone, 2% sucrose and 1% glucose.

3.3. Transformation

1. Sterile petioles are cut into 5–10 mm long segments using a scalpel and incubated in the *Agrobacterium* suspension in an empty petri plate for 10 to 30 min with gentle shaking. The suspension is then drained and the carrot explants are blotted dry on sterile filter paper and placed onto MS1D. Approximately 20–30 explants are placed on a 9-cm Petri plate and co-cultivated in the dark for 2–3 d (*see* **Note 5**).
2. Infected explants are subsequently rinsed in sterile H_2O, blotted dry, and placed on selective MS1D medium containing 1 mg/L PPT and 300 mg/L timentin (*see* **Fig. 1A**). Positive and negative controls are included. Positive controls are placed on medium lacking PPT whereas negative controls are noninfected with bacteria and placed on selective medium. Incubated at room temperature (22°C) under cool white fluorescent lights (450 μmol/m^2/s, 16 h/ d), all subsequent steps are incubated under these same conditions.
3. Explants are transferred to higher selection after 2 wk, using MS(1/2)D medium containing 10 mg/L PPT and 300 mg/L timentin. Explants are maintained on this medium, with transfers every 4 wk, until calli develop (*see* **Fig. 1B**). Somatic embryos typically begin to appear between 8 and 12 wk following infection. Nonembryogenic callus are maintained for up to 20 wk and discarded at that time if embryos have not developed (*see* **Fig. 1C**).
4. Somatic embryos (*see* **Fig. 1E**) are transferred to hormone-free MS medium containing 10 mg/L PPT and 300 mg/L timentin for regeneration (*see* **Fig. 1F**). Alternatively, the embryogenic calli are transferred into conical flasks (250 mL) containing 50 mL of liquid MS1/4D (50 mL) with 5 mg/L PPT and 150 mg/L timentin for initiating suspension cultures.
5. The embryos are subcultured to fresh medium every 2 wk. The embryos are transferred every month onto hormone-free MS with selection for regeneration (*see* **Fig. 1G**).
6. The tiny plantlets or small shoots are transferred to Magenta boxes containing hormone-free MS medium plus 10 mg/L PPT and 300 mg/L timentin (*see* **Fig. 1H**). Roots are induced quickly after transfer of shoots to larger containers, if they are not present prior to the initial transfer (*see* **Fig. 1I**).
7. The fully rooted plantlets are transferred to potting medium in 9 × 9 cm pots and placed in a growth chamber maintained at 26°C, 85% relative humidity, and a 16 h photoperiod (450 μmol/m^2/s) where they grow into full plants (*see* **Fig. 1K**).
8. Once the plants reach a size greater than 15 cm, they are tested for resistance to the herbicide Liberty. Leaves of carrot plants are painted with a 0.2 or 0.4% (w/v) Liberty solution and visually assessed for resistance (*see* **Fig. 1L**) (*see* **Note 6**). Visually healthy leaf tissue was collected from plants at this stage for PCR, Southern, Northern and Western analysis.
9. Effect of carrot cultivar on transformation frequency was measured as a proportion of infected explants that developed into individual Southern positive plants that grew on herbicide selection medium (*see* **Note 7**).
10. Once the transgenic carrots are larger than 20 cm in height they are transferred to 18-cm diameter pots and grown to maturity under the same conditions. The plants

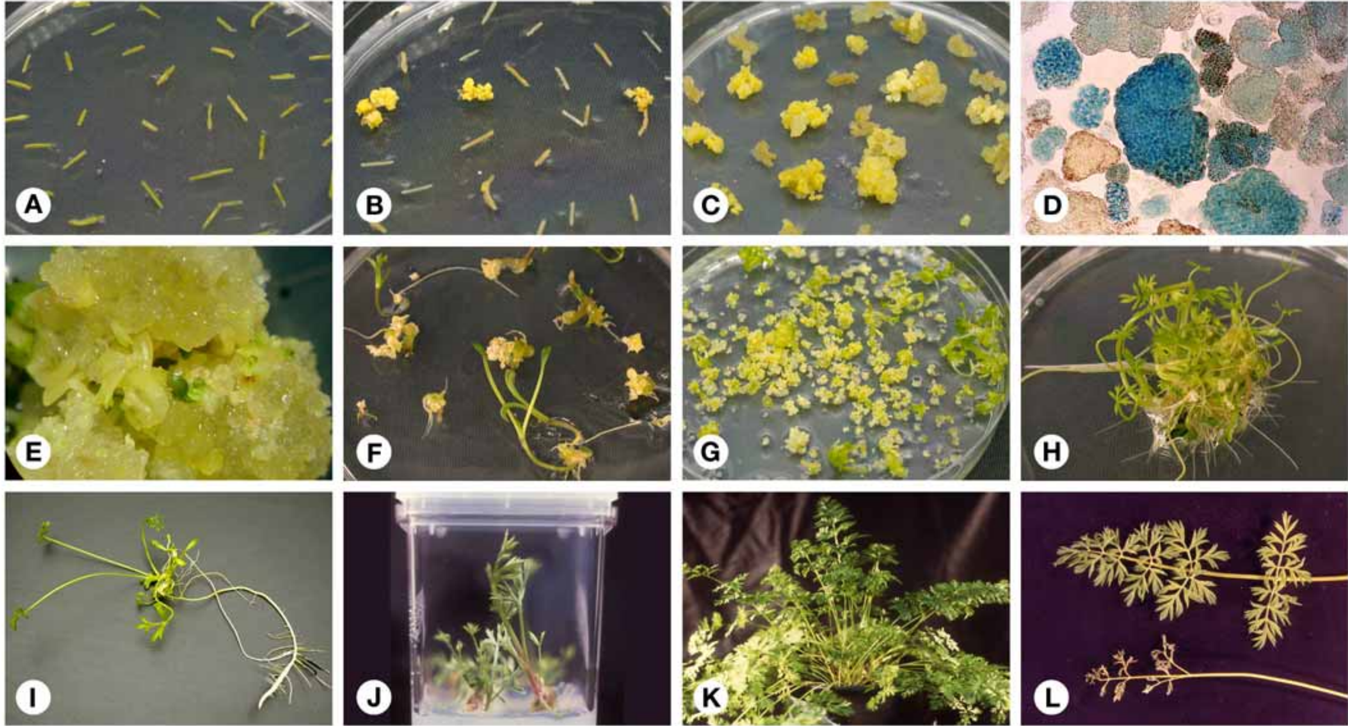

Fig.1.

are watered twice per wk (or as needed) and fertilized once per wk with a 20:20:20 fertilizer (500 mg/L). Pest problems are primarily from western flower thrips [*Frankliniella occidentalis* (Perg.)], which are difficult to control (*see* **Note 8**).

11. Carrots are a biennial plant and flower after their second growing season. To induce vernalization, the carrot plants at the 7–8 leaf stage are placed at 8°C for 10 wk *(8)*. The dead and dying foliage is removed and the plants are transferred back to the previous growing conditions. When flowering shoots are produced, the individual heads are encased in a glassine bag with 'blow flies' and tied off to prevent insect escape *(9)*. Seeds are then removed after 3–6 wk and stored desiccated at room temperature *(9)*.

3.4. Molecular Analysis of Transformants

1. Total genomic DNA is isolated from rooted plantlets using a protocol modified from Zang et al. *(9)*. A single plantlet or a leaflet, approx 50-mg fresh weight, is ground with a mortar and pestle under liquid nitrogen with a pinch of sea sand.
2. The macerated plant tissue is transferred to a 1.5-mL microcentrifuge tube to which 500 µL of pre-warmed (60°C) extraction buffer and 5 µL of dithiothreitol (DTT) stock are added. The mixture is vortexed for 30 s and incubated at 60°C for 60 min.
3. Following incubation, 500 µL of cold phenol:chloroform:isoamylalcohol is added and mixed by inversion and centrifuged at 7500*g* for 15 min.
4. The aqueous phase is removed and transferred to a fresh 1.5-mL tube, to which 500 µL of chloroform:isoamylalcohol is added, mixed and centrifuged at 7500*g* for 10 min.
5. The aqueous phase is transferred to another fresh 1.5-mL tube to which 250 µL of cold isopropanol is added, mixed and incubated at –20° C for no more than 30 min.
6. The mixture is then centrifuged at 18000*g* for 15 min and the supernatant drained, the pellet washed with 500 µL of 70% ethanol and re-centrifuged at 18,000*g* for 5 min.
7. The pellet is then drained and allowed to air dry before being resuspended in 50 µL of TE.

Fig. 1. **(A)** Transformation procedure and development of transgenic carrots. **(A)** Petiole explants following infection with *A. tumefaciens* and transfer to MS1D supplemented with 1 mg/L PPT. **(B)** Callus development from explants on MS (1/2)D medium supplemented with 10 mg/L PPT, 6 wk post infection with *Agrobacterium*. **(C)** Callus growth and early formation of somatic embryos on MS(1/2)D supplemented with 10 mg/L PPT, 12 wk post infection. **(D)** Callus fixed and displaying GUS positive phenotype. **(E)** Somatic embryo formation and the start of plantlet regeneration on MS with 10 mg/L PPT, 14 wk post infection. **(F)** Plantlet regeneration from somatic embryos on MS with 10 mg/L PPT, 18 wk post infection. **(G)** Plating of suspension cultures containing somatic embryos on MS with 10 mg/L PPT, 3 wk after plating. **(H)** Cluster of carrot plantlets on MS with 10 mg/L PPT. **(I)** Plantlet removed from a Magenta box. **(J)** Plantlet showing root development growing in a Magenta box on MS with 10 mg/L PPT. **(K)** Mature carrot plant, potted in soil and growing vigorously. **(L)** Transgenic carrot leaf treated with 0.4% Liberty solution (top) and control plant leaf (bottom). Photographs are of 9-cm diameter Petri dishes used for tissue culture.

8. The DNA is quantified after treating the sample with 5 µL RNAse A and incubated at room temperature for 1 h, at which point the OD_{260} is measured using a spectrophotometer. Once quantified, the remaining DNA can be stored at −20°C.
9. PCR: each reaction in 25 µL contains 100 ng of carrot DNA, 50 p*M* primers, 1 unit of Taq polymerase, and 1.5 m*M* $MgCl_2$ in 1X PCR buffer. The annealing temperature is 66°C for TLP and 35 cycles of 94°C for 30 s, 66°C for 50 s and 72°C for 90 s followed by a 5 min 72°C final extension.
10. 10 µL of the PCR product is run on a 1% agarose TAE gel and stained with ethidium bromide. The tlp PCR using the primers tlpF/tlpR produces a fragment of 691 bp, whereas the bar PCR using the primers barF/barR produces a fragment of 202 bp (*see* **Fig. 2A**).
11. Southern blotting is performed to identify unique transformation events; using 10 µg of total genomic DNA digested with *Hin*dIII, run on 1% agarose gel, transferred to a nylon membrane and hybridized using standard protocols with specifically designed bar and TLP DNA probes *(4)* (*see* **Fig. 2B**).
12. Western blotting is performed to ensure recombinant protein expression; using 200 µg of total proteins extracted, run on polyacrylamide gel, transferred to a nylon membrane, and probed with anti-bar and anti-TLP rabbit antibodies using standard protocols *(7)* (*see* **Fig. 2C**).

4. Notes

1. For sterilization, all water, media, and instruments are autoclaved at 15 psi for 15 min. A bead sterilizer is used for sterilization of instruments used in transformations and for callus transfer.
2. All water used in these experiments has a resistance of greater than 18 MΩ-cm and minimal organic content, referred to as H_2O.
3. Media performed the best when allowed to dry in the laminar flow hood for at least 24 h after pouring. This drying step reduced the amount of condensation formed on the plates and resulted in reduced fungal contamination.
4. Antibiotics and DL-PPT solutions are added to media after they are cooled to approx 50°C, and swirled to mix. 2,4-D can be added prior to sterilization as it is thermo-stable.
5. Carrot petioles are dissected on top of sterile filter paper and cut into the appropriate sizes. This alleviated some of the difficulties associated with the petioles dehydrating and sticking to smooth surfaces, such as a petri plate. Following dissection of each petiole (10–25 explants), they are immersed in the *Agrobacterium* solution. This caused some explants to have longer exposure to the inoculum; however, it was necessary to avoid the suberization of wound sites.
6. Some of the PPT-resistant plants still show some susceptibility to the 0.4% Liberty application; however, it is significantly less than the susceptibility of control plants. The minimum lethal concentration of Liberty to each plant needs to be ascertained by a preliminary experiment. The concentrations range from 0.1 to 0.4%. Cotton swabs are used to gently paint the herbicide solution over the leaf surface pre-marked with a water-proof ink or marker pen. Typical phytotoxicity symptoms developed after 7 d.

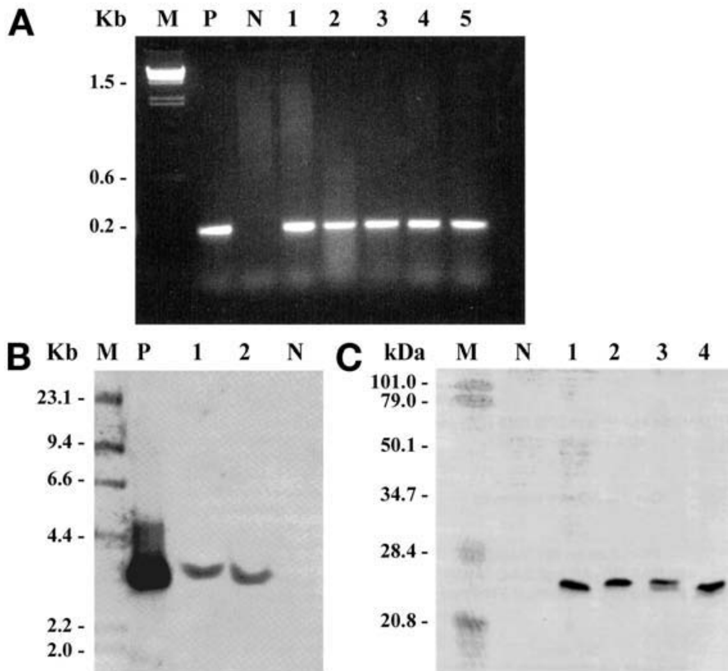

Fig. 2. Detection of transgenes in calli and plantlets by PCR analysis and Southern analysis and detection of TLP protein by Western analysis. **(A)** PCR analysis for the *bar* gene in transgenic tissues produced a band of 202 bp; M, molecular weight markers; P, positive control plasmid DNA; N, nontransformed control carrot DNA, *lanes 1–5* are sample lanes. **(B)** Southern analysis. Plant DNA extracts and plasmid controls were digested with *Hin*dIII and probed with PCR-amplified TLP coding region. **(C)** Western blot. Each lane was loaded with 25 μg protein, and probed with anti-TLP antibody. M, molecular weight markers; N, nontransformed tissue protein, transformed tissues showing a 23-kDA band.

7. There are significant differences in the transformation frequency depending on the cultivar selected. Danvers Half-long and Nantes Coreless have efficiencies greater than 3%, Nanco and HCM cultivars have efficiencies of less than 1% *(4)*. These efficiencies are the number of independent Southern-positive events from 100 explants.
8. Insect pests on carrot plants can be mimized with weekly applications of "Safer" insecticidal soap, according manufacturers instructions. In severe cases where thrips are visually detected, applications of active *Amblyseius cucumeris*, a predatory mite grown on a bran flake medium, are applied directly to infested carrot plants.

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2

Cassava (*Manihot esculenta* Crantz)

Weston Msikita, Uzoma Ihemere, Dimuth Siritunga, and Richard T. Sayre

Summary

During the last three years the generation of stably transformed cassava plants having value-added traits has become a reality. Currently, two *Agrobacterium*-mediated transformation systems are routinely used to engineer cassava. These systems use either somatic embryos or friable embryogenic calli. This paper presents detailed protocols for the transformation of cassava using primary somatic embryos. The effects of explant types, tissue culture conditions, and bacterial and plasmid related factors on transformation efficiency are discussed.

Key Words: *Agrobacterium tumefaciens*; apical leaves; auxiliary buds; cassava; direct embryogenesis; explant; friable embryogenic callus; genetic transformation; germinating somatic embryos; *Manihot esculenta* Crantz; shoot regeneration; somaclonal variation.

1. Introduction

The agronomic improvement of cassava (*Manihot esculenta* Crantz) using conventional breeding approaches has been hampered by a variety of factors including low production of flowers, apomixis *(1,2)*, a long reproduction cycle, limited seed set *(3)*, variability in ploidy number, and inbreeding depression *(4)*. Furthermore, cassava is most commonly propagated as genetically uniform, clonal stem cuttings. These factors make cassava an ideal target for genetic improvement via transgenic approaches involving clonal propagation. The recent development of reliable and, to some degree, genotype–independent methodologies for the transformation of cassava now makes it possible to consider directed strategies for the genetic improvement of cassava via transgenic approaches.

Recently, advancements in cassava transformation technology and its applications have been described in several review articles *(5–7)*. Both particle gun–mediated bombardment *(8)* and *Agrobacterium tumefaciens*-mediated gene transfer *(9–11)*

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have been used to engineer cassava. To date, *Agrobacterium*-mediated transformation of cassava has proven to be more successful than particle gun–mediated transformation *(6,9–15)*. This may be because Ti plasmid integration commonly occurs in transcriptionally active domains of the chromatin as well as to a reduced number of gene copies integrated relative to particle gun–mediated transformation *(6,14)*. To date, however, these hypotheses are only speculative as a result of the limited numbers of experiments reported using both systems.

The plant tissue types used for *Agrobacterium*-mediated transformation include shoots induced by organogenesis *(15)* and germinating somatic embryos (GSE) *(9,16)*. Direct shoot induction from cotyledons of somatic embryos (somatic cotyledons) has been used in both particle bombardment *(14)* and in *Agrobacterium*-mediated transformation *(17)*. However, plant regeneration efficiency is highly variable (5–70%) and genotypic dependent *(14)*. As a result of these cultivar-dependent differences a variety of tissues including auxiliary buds *(17–19)*, apical leaves *(10)*, and floral meristems *(20)* have been used for *Agrobacterium*-mediated transformation of cassava. Briefly, embryos are induced from callus tissues and directly inoculated with *Agrobacterium* for transformation. When using friable embryogenic callus (FEC), embryos are initiated and then transferred from Murashige and Skoog (MS)–based medium to Greshoff and Doy *(21)* basal medium supplemented with a high concentration of the auxins 4-amino-3,5,6-trichloropicolinic acid (picloram) or 2,4-dichlorophenoxyacetic acid (2,4-D) to initiate massive secondary embryo production. A disadvantage of the FEC system (compared to the direct somatic embryogenic system), however, is that it results in a high frequency of somaclonal variants *(22,23)*. In this paper we describe detailed protocols for the *Agrobacterium*-mediated transformation of cassava using GSEs. Embryos are produced from explant tissues cultured on semi-solid MS-based callus induction medium *(24)*. Embryos are then induced from callus tissues and directly inoculated with *Agrobacterium* for transformation. Selection for transformants is typically based on antibiotic resistance conferred by a gene present in the T-DNA. The transformation efficiency using our system ranges between 1 and 5% [defined as the frequency of polymerase chain reaction (PCR) transgene-positive plants per 100 cassava GSEs infected].

2. Materials

2.1. Plant Materials

Greenhouse grown cassava (MCol 2215, TMS 71173) plants are introduced in vitro by sterilizing the nodal segments of 4- to 6-mo-old plants with 20% (v/v) household bleach (sodium hypochlorite solution), 0.05% (v/v) Tween-20 (polyoxethylenesorbitan monolaurate) (Sigma-Aldrich, St. Louis, MO; cat. no. 63178), or 0.05% (v/v) Silwet L-77 (Polyalkyleneoxide) (Setre Chemical Company, 6075 Poplar Memphis, TN, cat. no. 38119).

2.2. **Agrobacterium** ***Strain and Constructs***

1. *Agrobacterium* LBA4404 (Invitrogen) was used in cassava transformation.
2. The main vector backbone used in our laboratory is pKYLX (*see* **Note 1**).

2.3. Stock Solutions

All prepared media stock solutions are filter sterilized using a 0.45 μm Cameo 25ES filter (Fisher Scientific) attached to a 60 mL BD syringe (Fisher Scientific) and are stored at 4°C. Plant hormones and antibiotics are supplied by Sigma-Aldrich.

1. α-Naphthaleneacetic acid (NAA, 0.02 mg/L) solution: 0.02 mg NAA in 1 L of ddH_2O. The NAA stock is made in 20% (v/v) 1 *N* NaOH. The stock can be stored at 4°C for 6 mo.
2. 6-Benzylaminopurine (BAP, 10 mg/L) solution: The BAP stock solution is made by dissolving in 20% (v/v) 1*N* NaOH and can be stored at 4°C for 6 mo.
3. Gibberelic acid solution (GA, 10 mg/L): The stock solution for GA is made by dissolving in 20% (v/v) 95% ethanol and can be stored at 4°C for 6 mo.
4. Thiamine (100 mg/L) solution: The thiamine-HCl (100 mg/L) stock is made by dissolving in distilled water. The stock is stored at 4°C for 6 mo.
5. 2,4-D (2,4-dichlorophenoxyacetic acid) solution: 0.5 mg/mL stock. The 2,4-D (0.5 mg/mL) stock is made by dissolving appropriate concentrations in 20% (v/v) 1 *N* NaOH . The stock is stored at 4°C for 6 mo.
6. 100X B5 vitamin solution: The 100X B5 vitamin stock solution was made by dissolving 10 g myo-inositol, 100 mg nicotinic acid, 100 mg pyridoxine-HCl, and 1 g thiamine-HCl in 1 L distilled water. The stock is stored at 4°C for a maximum of 6 mo.
7. Peters nutrient solution: 10% (w/v) nitrogen (ammonium sulfate), 50% (w/v) phosphorus (ammonium phosphate sulfite), and 10% (w/v) potassium (potassium sulfate) (J.R. Peters, Inc., Allentown Way, PA).
8. Acetosyringone solution: 50 m*M* acetosyringone (3, 5-dimethoxy-4 -hydroxyacetophenone) is made by dissolving 98 mg of acetosyringone with 10 mL of 95% ethanol. The stock solution is stored at –20°C.
9. Antibiotic solutions: The antibiotic (e.g., paromomycin and streptomycin) stock solutions are made by dissolving 20 mg antibiotic/mL in distilled water and filter sterilized using a 0.45 μm syringe filter. They are stored at –20°C. The antibiotics in the plant tissue culture media are added fresh to the media once it's cooled to 50°C each time media is made.

2.4. Plant Media

Four types of media are used in cassava transformation and in the subsequent recovery of putative transformants. With the exception of the rooting medium, all the media contain equivalent concentrations of MS salts and sucrose but differ in the concentrations and types of hormones included (*see* **Table 1**). All

Table 1

Reagent	Stock concentration	Final concentration	4E shoot multiplication/L	MS8 callus/somatic embryos induction/L	RM1 embryo maturation/L	17N rooting medium/L
MS salts			4.3 g	4.3 g	4.3 g	1.44 g
B5 Vitamins	100X	1X	–	10 mL	–	–
Sucrose		20 g/L	20 g	20 g	20 g	20 g
myo-inositol	8 g/L	100 mg/L	100 mg		100 mg	
BAP	10 mg/L	0.04 mg/L	4 mL	–	–	–
2,4-D	0.5 mg/ml	8 mg/L	–	16 mL	–	–
GA	10 mg/L	4E–0.05 mg/L RM1–1.0 mg/L 17N–0.01mg/L	5 mL	–	10 mL	1 mL
Thiamine-HCl	1 g/L	4E–10 mg/L RM1–10 mg/L 17N–6 mg/L	10 mL	–	10 mL	6 mL
NAA	10 mg/L	4E–0.02 mg/L RM1–0.01mg/L 17N–0.01 mg/L	2 mL	–	1 mL	1 mL
$CuSO_4$	1 m*M*	1.88 µ*M*	–	1.88 mL	–	–
Casein hydrolysate		50 mg/L	–	50 mg	–	–
Peters nutrient	5 g/L	25 mg/L		–	–	5 mL
pH		5.7	5.7	5.7	5.7	5.7
Agar		8 g/L	8 g	–	–	8 g
Phytagel		2 g/L	–	2 g	2 g	–

media are adjusted to pH 5.7, autoclaved (20 min at 1.06 kg/cm^2 pressure), cooled to 50°C and dispensed into 100 × 15-mm Petri dishes. Prepared media are stored at 4°C. Final concentrations for all components are listed in Table 1.

1. 4E-Micropropagation medium: 1X MS salts (4.3 g), sucrose (20 g), myo-inositol (100 mg), and 2.0 mL of NAA solution are dissolved in 800 mL de-ionized water. The pH is then adjusted to 5.7 using 1 *N* potassium hydroxide (KOH) or 1 *N* hydrochloric acid (HCl) and the final volume is brought to 1 L. Add 8 g of agar (Fisher Scientific) after pH adjustment. The media is then autoclaved and 4 mL of BAP solution, 5 mL of GA solution, and 10 mL of thiamine-HCl solution are added after the medium has cooled to 50°C (*see* **Note 2**).
2. MS8-callus/somatic embryo induction media: 1X MS salts (4.3 g) and sucrose (20 g) are dissolved in 800 mL of de-ionized water. Add 1.88 mL of copper sulfate solution, 16 mL of 2,4-D solution, and 50 mg of casein hydrolysate. The pH is adjusted to 5.7 using 1 *N* KOH or 1 *N* HCl, and the final volume is brought to one liter before adding 2.0 g of Phytagel prior to autoclaving. After autoclaving, add 10 mL of B5 vitamin solution when the medium cools down to about 50°C (*see* **Note 3**).
3. RM1-embryo maturation medium: 1X MS salts (4.3 g), sucrose (20 g), myo-inositol (100 mg), and 1.0 mL of NAA solution are dissolved in 800 mL of de-ionized water. The pH is adjusted to 5.7 as described above and the final volume is brought to 1L. Add 2 g of phytagel and autoclave. Add 10 mL of GA solution and 10 mL of thiamine solution after autoclaving when the medium has cooled to 50°C (final concentrations listed in Table 1) (*see* **Note 4**).
4. 17N-rooting medium: Add $^1/_3$ MS salts (1.44 g), sucrose (20 g), 1.0 mL of NAA solution and 5 mL of Peters Nutrient fertilizer to 800 mL of deionized water. Adjust the pH to 5.7 as described above and bring to a final volume of 1 L. Add 8.0 g of agar prior to autoclaving. Add 1.0 mL of GA solution and 6.0 mL of thiamine solution after autoclaving when the medium cools down to 50°C (final concentrations listed in **Table 1**). Fifty milliliters of the medium is prepared and dispensed in magenta boxes. Alternatively, 4E media supplemented with 0.5 g charcoal/L can be used for rooting.

2.5. Agrobacterium *Media*

1. YM-*Agrobacterium* medium: One liter contains 0.4 g yeast extract and 10 g mannitol. Salts are present in the following final concentrations: 1.7 m*M* NaCl, 0.8 m*M* $MgSO_4$, and 2.2 m*M* K_2HPO_4, pH 7.0. For solid medium, add 15 g /L agar before autoclaving. Appropriate antibiotics are added as required after autoclaving and cooling to 50°C.
2. Co-culture medium: 1X MS basal salt medium devoid of nitrogen (*see* **Table 1**), but supplemented with 10 g/L glucose and 10 g/L galactose and the pH is adjusted to 5.5.

2.6. Other Reagents and Supplies

1. Sterilization solution: 20% (v/v) household bleach (sodium hypochlorite solution), 0.05% (v/v) Tween-20 (Polyoxethylenesorbitan monolaurate) (Sigma), or 0.05% (v/v) Silwet L-77 (Polyalkyleneoxide) (Setre Chemical Company).

2. No. 1 Whatman filter paper (Fisher Scientific).
3. Magenta box: 77 × 77 × 97 mm (Sigma).
4. BD1M needle: 38-mm long (Fisher Scientific).
5. No. 11 surgical blade (Fisher Scientific).

3. Methods

Various types of meristematic tissues are used to initiate callus, which is subsequently transferred to embryo-induction medium, and to transform developing embryos with *A. tumefaciens*. The explants have included apical leaves, undifferentiated callus tissue, germinating somatic embryos, auxiliary buds, and floral tissues (*see* **Fig. 1**). Because cassava rarely flowers, floral parts are not frequently used for cassava transformation. To date, only a few cassava cultivars have been reported to have been transformed. Below, we provide detailed descriptions of the explant tissues commonly used in cassava transformation. A flow diagram describing the overall scheme for *Agrobacterium*-mediated transformation of cassava is presented in **Fig. 2**.

3.1. Preparation of Explants for Transformation

3.1.1. Surface Sterilization, Preparation of Apical Leaf and Initiation of In Vitro Plants

1. Sterile in vitro cassava plants are initiated from immature stem cuttings (located 2–10 cm from the shoot apex) obtained from 4- to 6-mo-old greenhouse grown plants.
2. Stems are excised, and divided into pieces with two to three nodes.
3. The pieces are then surface sterilized using sterilization solution, and maintained on a shaker (250 rpm) for 10 min.
4. The stem pieces are then rinsed three times with sterilized distilled water and blot-dried on sterile No. 1 Whatman filter paper.
5. The stem pieces are cultured by inserting the basal stem portion into Magenta boxes containing 50 mL semi-solid MS shoot multiplication medium (4E).
6. Cultures are incubated at 26°C under a 12-h light/dark regime provided by cool-white fluorescent lamps (40 µmole/m^2/s) until young actively growing apical leaves emerge (about 3 wk after culture).
7. At this stage the apical leaves are ready for initiating embryogenic cultures (*see* **Subheading 3.1.2.**) or direct transformation (*see* **Subheading 3.3.**). The in vitro plants can supply apical leaves for up to 3 mo.

3.1.2. Preparation of Undifferentiated Callus Tissue

1. Young actively growing apical leaves (*see* **Fig. 1A**) are excised from clean (non-contaminated) cultures, placed onto a sterile filter paper and wounded (5–7 pin holes) using a sterile 38- mm long BD1M needle.
2. Wounded apical leaves are then placed with the adaxial surface on MS8 medium to initiate callus cultures.

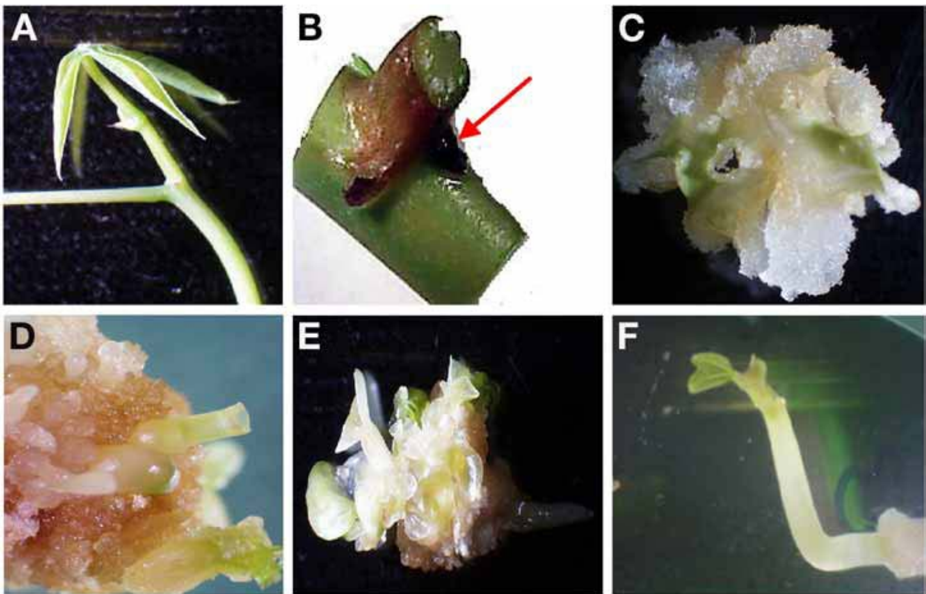

Fig. 1. In vitro and in vivo cassava tissues: (**A**) apical leaf, (**B**) axillary bud, (**C**) callus tissue, (**D**) embryogenic callus tissue, (**E**) germinating somatic embryos, (**F**) a developing plantlet. Arrow indicates axillary bud.

3. Small embryos will typically develop from 3- to 4-wk old callus cultures (*see* **Fig. 1D**) and may be used directly for transformation or transferred to embryo maturation (*see* **Fig. 2**) (RM1) medium to develop germinating somatic embryos (Section 3.1.3).

3.1.3. Preparation of Germinating Somatic Embryos

1. Three- to four-wk old embryoid callus tissues (*see* **Subheading 3.1.2.**) are transferred to embryo maturation medium (RM1) and incubated at 26ºC under a 12-hr light/dark regime.
2. After 3- to 4-wk, germinated somatic embryos are excised and briefly washed in MS media containing 0.05% (v/v) Tween-20.
3. The germinated somatic embryos are then cut into pieces approxi 25–50 mm^2 and are wounded using a sterile 38-mm long BD1M needle prior to inoculation with *Agrobacterium* (*see* **Fig. 1**).

3.1.4. Preparation of Auxiliary Buds

1. Nodal stem cuttings with two to three auxiliary buds are excised from immature stem portions and surface sterilized as described in **Subheading 3.1.1.**
2. Buds are excised using a No 10 surgical blade. A small incision is made on the surface of the excised bud.

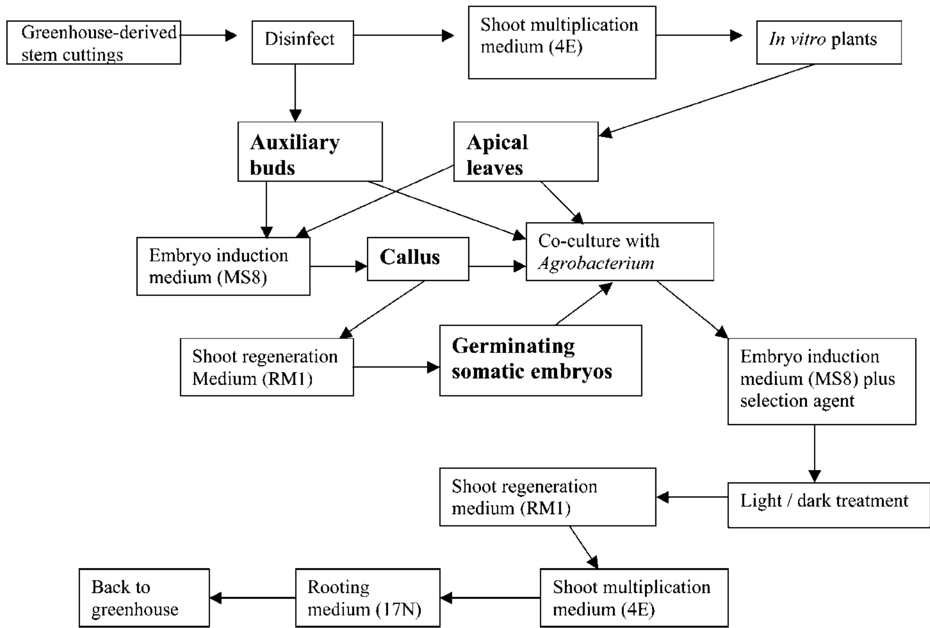

Fig. 2. Overall scheme of *Agrobacterium*-mediated transformation of cassava. Explant types are shown in bold.

3. At this stage the buds may be used to initiate callus cultures (**Fig. 2**). To do so, the buds are cultured on MS8 medium supplemented with 16 mg/L (MS16, two times the regular amount) 2,4-D.
4. Alternatively, the buds may be directly inoculated with *Agrobacterium* and inoculated on solid MS16 medium (*see* **Subheading 3.3.**).

3.2. Agrobacterium *Preparation*

1. *Agrobacterium* strain LBA4404 (*see* **Note 5**) containing the vector of interest is streaked and grown for 2 d on solid YM medium supplemented with 100 mg/L streptomycin and 6 mg/L tetracycline (or relevant antibiotic according to the plasmid of choice). Cultures are incubated at 28°C.
2. After 2 d, single colonies are used to inoculate 20 mL of liquid YM medium supplemented with 200 μ*M* acetosyringone, 100 mg/L streptomycin, 6 mg/L tetracycline (or the antibiotics used depend on the plasmid used), and incubated at 28°C on a shaker at 250 rpm for 2 d.
3. After 2 d growth, the bacteria are pelleted by centrifugation (10,500*g*) for 7 min, and resuspended in 20 mL of MS basal salt (co-culture) medium devoid of nitrogen, but supplemented with 1% (w/v) of glucose and 1% (w/v) of galactose, and the pH adjusted to 5.5.
4. The optical density (OD_{600}) of the bacterial suspension is measured and adjusted to 0.5 (approx 0.5×10^9 cfu/mL) using a spectrophotometer.
5. At this stage the bacteria are ready for inoculating the explants.

3.3. Explant Inoculation and Cocultivation

1. Following wounding explants are transferred onto solid MS8 medium supplemented with 200 μ*M* acetosyringone.
2. Two drops of the bacterial suspension in liquid MS [to which is added 0.05% (v/v) Tween-20] are applied to the explant tissue, and the tissue is incubated in the dark for 2 d at 24–28°C. Subsequently, the explants are transferred to a new MS8 plate with 200 μ*M* acetosyringone for another 3 d co-cultivation (*see* **Note 6**).
3. Following co-cultivation with *A. tumefaciens*, explants are transferred to selection media (MS8 supplemented appropriate selective agent according to the vector used and 500 mg/L of carbenicillin) (*see* **Note 7**).
4. To induce embryogenesis explants are incubated in the dark for 4 wk. For germplasm maintenance explants are transferred directly to a 12-h light/dark regime under 40 μmol/m^2/s lighting intensity after 1 wk dark incubation (*see* **Note 8**).
5. Explants are maintained on selection medium for 4 to 6 wk. Somatic embryos produced on selection media are transferred to selective RM1 media (RM1 supplemented appropriate selective agent according to the vector used and 500 mg/L of carbenicilin) on which the embryos convert to plants.
6. Plantlets are regenerated following successive transfers of the inoculated nodal segment explants onto shoot induction (4 wk), shoot regeneration (4 wk), and shoot multiplication media (4 wk) (*see* **Note 9**).

3.4. Selection and Regeneration of Putative Transformants

1. Selection of putative transformants follows successive transfers of the inoculated explants onto somatic embryo induction (MS8), and embryo maturation (RM1) media supplemented with appropriate selection agents plus carbenicillin (500 mg/L) to kill *A. tumefaciens*.
2. Cultures are examined biweekly under a dissecting microscope to check for presence of embryos (*see* **Fig. 1D**) ready for transfer to maturation medium (RM1).
3. To transfer the embryos, a sterile needle is used to pick the embryos which are aseptically placed on maturation medium.
4. Sequentially, embryos are transferred from selection media to media free of the selection agent until shoots have elongated to about 1 cm.
5. Elongated shoots are excised and transferred to shoot multiplication medium (4E). Cultures are incubated at 26°C under a 12-h light/dark regime provided by cool-white fluorescent lamps (40 μmole/m^2/s) for 4 wk to allow the plants to grow to sufficient size for root induction.
6. From 4E medium, shoots are excised and cultured on rooting medium (17N).
7. Root formation takes approx 2–3 wk after which the plants are transplanted to soil. In vitro grown plants maintained on 4E medium are used for transgene analysis.

3.5. Greenhouse Care of Transgenic Plants

1. Transgenic plants that have been grown in vitro for 1 mo are transferred to sterile soil (3:1 soil/vermiculite) and grown in the greenhouse under natural lighting conditions and a temperature range of 22–30°C. The transferred plants are covered with transparent plastic bags to maintain high humidity.

2. The bags are removed after 2–4 wk to expose the plants to normal environment.
3. The transformed plants are now treated like the wild-type cassava plants by watering three times a week.

4. Notes

1. We have typically used the binary plasmids pKYLX or pBI121 in our laboratory for cassava transformation (*9–11*). Using *Agrobacterium* strains harboring the binary plasmids having T-DNA inserts ranging from 3.5 to 5.5 kb, we achieved transformation rates between 0.5 and 1.0% when using cassava apical leaves and between 2.4 and 5.0% when using germinated somatic embryos. The T-DNA included the *npt*II selectable marker driven by the NOS promoter. Transformants were selected using 75 mg/L paromomycin and confirmed by PCR analysis of the integrated transgene.
2. Micropropagation medium (4E) is designed not only to quickly multiply the plantlets but also to maintain the explants for a long period of time prior to subculture.
3. A prominent feature of the callus induction medium (MS8) is the addition of B5 vitamins (*7*), copper sulfate and casein hydrolysate (*see* **Table 1**). Copper sulfate is reported to increase the number of primary and secondary embryos (*7*).
4. The embryo maturation medium (RM1) contains no auxins or copper sulfate.
5. Other *Agrobacterium* strains such as C58 and EHA101 could be used for transformation. However, we found that LBA4404 worked better for our transformation system.
6. Co-culture of *A. tumefaciens* with the explant in liquid medium (*see* **Subheading 3.2.1.**) works well for large sized explants such as callus tissues and clumps of germinating somatic embryos. Small sized, and fragile explants such as wounded apical leaves and auxiliary buds respond better when co-cultured with *A. tumefaciens* on solid MS medium (*see* **Subheading 3.2.2.**).
7. The type of selection agent used in the medium is determined by the marker gene in the plasmid construct. Marker genes used in our plasmid constructs have included neomycin phosphotransferase II (*npt* II) which confers resistance to aminoglycoside antibiotics such as kanamycin and paromomycin (*25*), and phosphomannose isomerase (PMI) which catalyzes the reversible inter-conversion of mannose 6-phosphate to fructose 6-phosphate (*26*). Plant cells lacking the PMI gene are incapable of surviving on synthetic medium containing mannose as a carbon source (*26*). The embryo induction and embryo maturation selection media used contain paromomycin (75 mg/L), an analog of kanamycin or mannose (10 g/L, w/v) as selection agents, and carbenicillin (500 mg/L) to kill *A. tumefaciens.*
8. Light is reported to have a significant effect on cassava plant regeneration and to promote gene transfer from *A. tumefaciens* to plant cells (*27*).
9. Transformation efficiency (TE) is a quantitative measurement of the integration of the gene of interest into the host plant genome. TE may be measured by the number of desirable events (trait under selection, e.g., herbicide resistance) recovered per 100 explant pieces inoculated (*5*). In this paper, transformation efficiency refers to the number of PCR amplified transgene-confirmed positive plants per total number of explants inoculated (*23*).

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Weston Msikita and Uzoma Ihemere contributed equally to this document.

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3

Potato (*Solanum tuberosum* L.)

Steve Millam

Summary

Potato (*Solanum tuberosum* L.) is a globally important crop plant producing high yields of nutritionally valuable food in the form of tubers. It has been the focus of substantial study because of its use both as a staple food crop and as a potentially significant source of compounds of interest. This has included the development and application of transgenic technology for introducing novel traits of fundamental and applied interest. This chapter describes a rapid, efficient, and cost-effective system for the routine transformation of this crop plant at rates above 40% efficiency, calculated as the mean number of Southern blot–confirmed independent transgenics per number of internodal explants originally plated. Internodal sections are co-cultivated with *Agrobacterium tumefaciens* and subjected to a two-stage callus induction/shoot outgrowth system under kanamycin selection. Shoot regeneration rates are high using the described method, and excised independent shoots rooting from the cut end of the stem after two further subcultures on kanamycin are 95% certain to be transformed. The transgenic status can be confirmed by molecular analysis and the plants grown on for tuber production enabling a wide spectrum of further studies.

Key Words: Callus induction; internodal explant; kanamycin selection; potato; *Solanum tuberosum*; tuber.

1. Introduction

Potato was among the first plant species to be transformed, with direct evidence of transformation reported by Ooms et al. in 1986 *(1)* using the cultivar Desiree regenerated from *Agrobacterium rhizogenes* infected tissue. Transformations of cultivars Desiree and Bintje using *Agrobacterium tumefaciens* were made by Stiekma et al. *(2)*. de Block *(3)* reported a genotype-independent method for transformation using leaf discs as the target tissue. Visser et al. *(4)* published a two-stage regeneration and transformation method using stem and leaf explants that is the basis for many protocols used today. The relative ease of shoot regeneration from a range of tissues of potato (e.g., stem section, leaf,

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petiole, tuber disc) underpins the development of the systems employed for transformation in this species.

Most protocols in use today employ a two-step regeneration procedure, with a callus induction stage followed by a shoot outgrowth stage. The callus induction stage is often minimized to prevent the high incidence of somaclonal variation reported in potato *(5)*. This initial stage is often facilitated by treatment of explants with zeatin or zeatin riboside (1–5 mg/L) in conjunction with low levels of auxin, typically 0.1–0.2 mg/L α-naphthalene acetic acid (NAA) or indole acetic acid (IAA). The second stage often has the zeatin level reduced by 20% and the auxin level reduced by a factor of 10, plus the addition of gibberellin to stimulate shoot outgrowth. Regeneration rates per explant can be high, with the first shoots appearing after 4–6 wk and after 10–12 wk culture over 10 shoots per explant is not uncommon. The use of kanamycin or hygromycin as a selectable marker enables an easy visual discriminatory analysis, as following excision of putative independent transgenic shoots, plantlets containing the antibiotic resistance gene will clearly root from the cut stem base, and nontransgenics will either not root or will root from adventitious nodes. Transformation efficiencies vary between cultivars and constructs used, but rates of between 40 and 100% efficiency calculated as number of independent confirmed transgenic plants produced over the number of explants originally plated can reproducibly be recorded from cultivar Desiree.

Although potato is generally tetraploid ($2n = 4x = 48$), dihaploid ($2n = 2x = 24$) lines exist and can also be used in transformation studies using the same protocols. Additionally, related wild diploid species can also be transformed using this method *(6)*. Though many of the initial reports were based on model cultivars the range of genotypes successfully transformed has been widened. Studies on screening the potential for transformation of a range of cultivars have been made *(5,7)*, interestingly showing little correlation between regeneration efficiency and transformation potential. In terms of targets for transgenic manipulation, in recent years potato has been a focus of study for the development of transgenics imparting such characters as pest and disease resistance *(8,9)*, improved tuber quality *(10,11)* and modified starch production *(12)*.

2. Materials

2.1. Plant Material

Potato cv. Desiree in vitro microplants (*see* **Note 1**) (obtained from the Scottish Agricultural Science Agency [SASA], http://www.sasa.gov.uk/ or other verified sources). It is important to ensure virus free starting material. Alternatively, one can establish in vitro plants in culture from sprouting tubers, or less commonly from glasshouse grown plants.

2.2. Stock Growth Regulator Solutions

Enough stock growth regulator solutions for 16 × 250 mL of complete media are made up in advance. The solutions are filter sterilized as above, aliquoted into sterile Eppendorf tubes, and can be stored at −20°C for 6 mo (*see* **Note 2**).

1. α-Naphthalene acetic acid (NAA) (Duchefa): Made as 1 mg/mL stocks by dissolving 20 mg of NAA in 1 mL 1 *N* NaOH and making up to volume with 19 mL of distilled water. Stock solution can be stored at 4°C for 6 wk.
2. Gibberellic acid (GA_3) (Duchefa): Made as 1 mg/mL stocks by dissolving 20 mg of GA_3 in 20 mL 50% (v:v) ethanol:distilled water. Stock solution can be stored at 4°C for 6 wk.
3. Zeatin riboside (ZNR) (Duchefa): Made as 1 mg/mL stocks by dissolving 20 mg of ZNR in 1 mL of 1 *N* NaOH and making up to volume with 19 mL of distilled water. Stock solution can be stored at −20°C for 6 mo.

2.3. Stock Antibiotics Solutions

1. Cefotaxime (Claforan®, Cefotaxime powder, Roussel, Uxbridge, England): Prepared as 125 mg/mL stock solution in water, filter-sterilized, and can be stored at −20°C for 6 mo.
2. Kanamcyin monosulphate (Duchefa): Prepared as 50 mg/mL stocks dissolved in water, filter-sterilized, and can be stored at −20°C for 6 mo.
3. Rifampicin (Sigma): Prepared as 50 mg/mL stock in methanol, (not filter-sterilized), and can be stored at –20°C for 6 mo.

2.4. Media

All media is prepared as 250 mL aliquots in Durand bottles (VWR) autoclaved for 20 min at 121°C, and filter-sterilized antibiotics. Growth regulator formulations are added to the media prior to pouring 10 × 9-cm petri plates per bottle in a laminar flow cabinet.

1. Stock plant material medium (BM): 1X Murashige and Skoog (MS) *(13)* basal medium plus vitamin mixture powder (Duchefa, product no. M022), 20 g/L sucrose, to 1.0 L with distilled water, pH to 5.8. Add 8.0 g/L Micro agar, autoclave, and store at 4°C.
2. MS20 medium: A liquid form of BM, same formulation as above with no agar added.
3. Co-cultivation medium (CM): BM plus 0.2 mg/L of NAA, 0.02 mg/L of GA_3, 2.5 mg/L of zeatin riboside (*see* **Note 2**), and 8 g/L Micro agar.
4. First stage regeneration medium (CMC): CM plus 500 mg/L of cefotaxime (filter-sterilized).
5. Second stage regeneration medium (CMCK): BM plus 0.02 mg/L of NAA, 0.02 mg/L of GA_3, 2 mg/L of zeatin riboside, 500 mg/L of cefotaxime (filter-sterilized), 50 mg/L of kanamycin (filter-sterilized).
6. Selection medium (SM): BM plus 500 mg/L of cefotaxime (filter-sterilized) and 50 mg/L of kanamycin (filter-sterilized) (*see* **Note 3**).

7. Luria Broth (LB) media: 10 g/L of tryptone; 10 g/L of yeast extract; 10 g/L of NaCl, pH 7.5; 18 g/L of Agar.

2.5. Bacterial Strains and Vector

1. *Agrobacterium tumefaciens* strain LBA4404.
2. DNA construct: pBIN19 derivative binary vector (*see* **Note 4**). Vector was introduced into LBA4404 by electroporation (*see* **Chapter 3**, Volume 1). Transformed cells are selected for resistance on LB plus 100 mg/L of kanamycin and rifampicin (*see* **Note 5**). *Agrobacterium* stocks should be maintained as glycerol stocks held at −80°C.

2.6. Other Supplies and Solutions

1. Sterilization solution: 10% Domestos (Lever Brothers, UK) which contains 1.0% sodium hypochlorite as the active ingredient.
2. Compost mix: Irish moss peat (bedding grade), 1200 L; Pavoir sand, 100 L; limestone (magnesium), 2.5 kg; limestone (calcium), 2.5 kg; sincrostart base fertilizer (William Sinclair, Lincoln, UK), 1.5 kg; Celcote wetting agent (LBS Horticulture, Lancashire, UK), 1.5 kg; Perlite, 100 L; Osmocote mini-controlled release fertilizer (Scotts, UK), 2 kg; Intercept insecticide (Bayer), 390 g.

3. Methods

3.1. Establishment of In Vitro Stock Plants

All in vitro culture are carried out under environmental conditions of 18–22°C, 16-h light (80–110 μE/m^2/s) 8 h (*see* **Note 6**).

3.1.1. Establish In Vitro Plants From Sprouting Tubers

1. Excise shoots from spouting tubers and wash them in running water.
2. Immerse shoots in 70% ethanol with 2 drops of Tween-20 for 1 min, followed by 15 min in 10% Domestos and 5 subsequent washes in sterile water prior to establishing on BM.
3. Nodal and shoot tip cultures are subcultured onto 90-mm Petri-dishes or Magenta vessels containing BM (*see* **Note 7**).

3.1.2. Establish In Vitro Plants From Greenhouse Grown Plants

1. Nodes and shoot tips from actively-growing plants, free from any pest or disease, are excised and immediately washed in running water.
2. The nodes and shoots tips can be trimmed to size (5–10 mm) then rinsed in 70% ethanol with 2 drops of Tween-20 for 1 min, followed by 15 min in 10% Domestos and 5 subsequent washes in sterile water prior to establishing on BM.
3. Nodal and shoot tip cultures are subcultured onto 90-mm Petri-dishes or Magenta vessels containing BM (*see* **Note 7**).

3.2. *Agrobacterium* Preparation

1. A 5-mL starter culture is initiated from either a glycerol stock or plate culture comprising LB medium with 100 mg/L each of kanamycin and rifampicin.

2. The *Agrobacterium* is grown up overnight at 28°C on a shaker (200 rpm) in 5 mL LB medium containing rifampicin and kanamycin at concentrations of 100 mg/L each.
3. After overnight incubation, the 5 mL culture is transferred to 50 mL of LB plus appropriate antibiotics in a 250-mL flask and cultured under the same conditions.
4. Following a further overnight incubation, the optical density (OD) of the *Agrobacterium* suspension at 600 nm recorded (*see* **Note 8**).
5. The culture is transferred to a sterile tube and spun at 700–1000*g* for 15 mins and then pellet resuspended in 15 mL of MS20.
6. At this stage, 5 mL of the culture can be taken and used to make 1 mL glycerol stocks, 650 µL of culture medium to 350 µL of 50% Glycerol (v:v) and the samples flash frozen and stored at −80°C.

3.3. Inoculation, Co-Cultivation, and Regeneration

1. Internodal sections approximately 10-mm length and no less than 2.5-mm wide excised from 3- to 4-wk old potato stock plants (*see* **Note 9**).
2. Explants (up to 30 per plate) are placed into Petri dishes containing 15 mL of MS20 to avoid dessication (*see* **Note 10**).
3. Explants inoculated with 1 mL of the 15 mL *Agrobacterium* suspension from **Subheading 3.2.6.** per 90-mm Petri dish, dishes sealed with Nescofilm and placed on orbital shaker at 50 rpm for 45 min at 22°C (*see* **Note 11**).
4. *Agrobacterium* suspension poured off into a container for inactivation (*see* **Note 12**).
5. Explants are gently blotted using sterile filter paper and plated onto CM medium (30 explants per Petri dish). Plates are sealed and co-cultivated at 18–22°C, in low light conditions (20 µE/m^2/s) for 48 h (*see* **Note 13**).
6. Following co-cultivation period explants are subcultured onto CMC regeneration media, no more than 10 explants per plate (*see* **Note 14**). The plates are incubated at 18–22°C, in full light conditions (80–110 µE/m^2/s).
7. Explants are subcultured onto CMCK medium after 12 d (*see* **Note 15**).
8. Explants are further subcultured onto freshly prepared plates of CMCK at 14-d intervals (*see* **Note 16**).
9. Callus and shoot formation occurs after 4 wk and continues for several weeks (*see* **Fig. 1A**). At approx the third transfer onto fresh CMCK, carefully excise developing shoots (5–10 mm in size) and plate onto selection medium (SM) (*see* **Note 17**).
10. Continue transferring explants at 14-d intervals and excising further shoots at transfer of explants taking care to ensure that shoots taken are independent lines (*see* **Note 17**).

3.4. Selection and Further Growth of Transgenic Shoots

1. After 14 d on SM, remove surviving shoots (those with roots derived from cut ends of shoots only (*see* **Note 18** and **Fig. 1B**). Excise 10–15 mm shoot tip explants and replate onto SM for second selection.
2. Those plants rooting from cut end following this stage can be considered transformed and further subcultured onto SM for generating material for glasshouse culture.

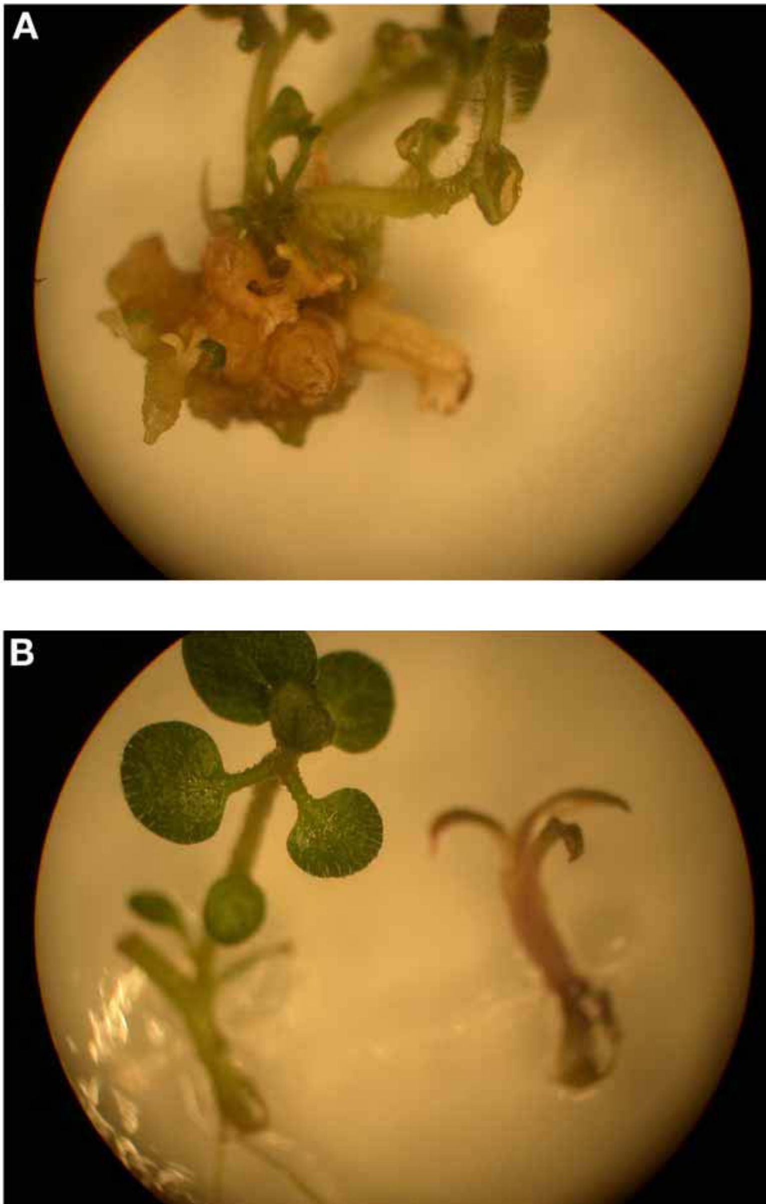

Fig. 1. (A) Multiple shoot formation from 5 mm internodal explant of Desiree after 6 wk in culture. Scale ×3. (**B**) Selection of putative excised shoots on 50 mg/L kanamycin for 14 d, plantlet on left shows rooting and is a putative positive, plant on right has no rooting and can be rejected as a negative. Scale ×3.

3. Well-rooted plantlets at the 4–5 node stage can be planted into compost mix in a 3-L size pot in a glasshouse (*see* **Note 19**) at 15–20°C (150 $\mu E/m^2/s$ light intensity and 16-h light photo period), by washing off the excess agar and planting into moist compost, watering in and keeping humid for 2 d (*see* **Fig. 2A**).
4. By carefully following these methods 100% transplant rates can routinely be obtained. Actively growing plants should be fed a liquid fertilizer (1:1:1 N:P:K) applied to the compost at weekly intervals during the flowering period. Harvest rates of 7–10 tubers per plant are normal when grown under the above conditions.
5. Transgenic lines can be maintained in vitro as cataloged microplants, or as tuber stocks derived from microplants and stored in appropriate low temperature (4°C, dark) containment stores (*see* **Note 20**).

4. Notes

1. Desiree was one of the first varieties transformed, and is still widely used as a model variety. The transformation system described has been successfully applied to a wide range of cultivars, including recalcitrant processing lines such as "Saturna" and wild species.
2. Example of Stock Plant Growth Regulator formulation preparation. CM formulation, for 16 × 250 mL aliquots medium (i.e., 4 L medium) = 0.8 mL NAA stock growth regulator solution, 0.8 mL GA_3 stock growth regulator solution, 10.00 mL zeatin riboside stock growth regulator solution, 5.12 mL water (total 16 mL; i.e., 1 mL/250 mL complete formulation) filter-sterilized, aliquoted into 1-mL sterile eppendorf tubes and stored at −20°C. These stocks can be kept for up to 6 mo.
3. In this example the selectable marker used is kanamycin where selection levels previously used are 50–100 mg/L. In the case of hygromycin, the level of selection should be determined empirically per cultivar used, but will be less than that used for kanamycin, often as low as 16–25 mg/L. Other markers have been used for potato transformation including nonantibiotic approaches such as galactose mediated by xylose isomerase ***(14)*** or a UDP-glucose: galactose-1-phosphate uridyltransferase gene ***(15)***.
4. A wide range of *Agrobacterium* vectors have been used for transformation of potato (for an extensive guide to available *Agrobacterium* vectors *see* Hellens et al. ***16***).
5. Though antibiotic resistance systems work efficiently, attention should be drawn to the use of such systems being phased-out under EU regulations, thus other systems should be investigated (*see* **Note 4**).
6. This enables a 3- to 4- wk cycling period for nodal sections grown for stock plant material of most cultivars of potato tested. For other related species such as *S. phureja* a longer culture period of up to 6–8 wk may be necessary to generate plants with enough internodes. For successful development of potato in vitro, the culture vessels need to be vented to enable adequate gaseous exchange.
7. The use of vented Magenta GA7 vessels, Suncaps (Sigma) or Vitro Vent Vessels (Duchefa) will facilitate this. For culture in Petri dishes, the plates should be sealed with Nescofilm and a 30-mm slit made in the seal, which is then covered with micropore tape.

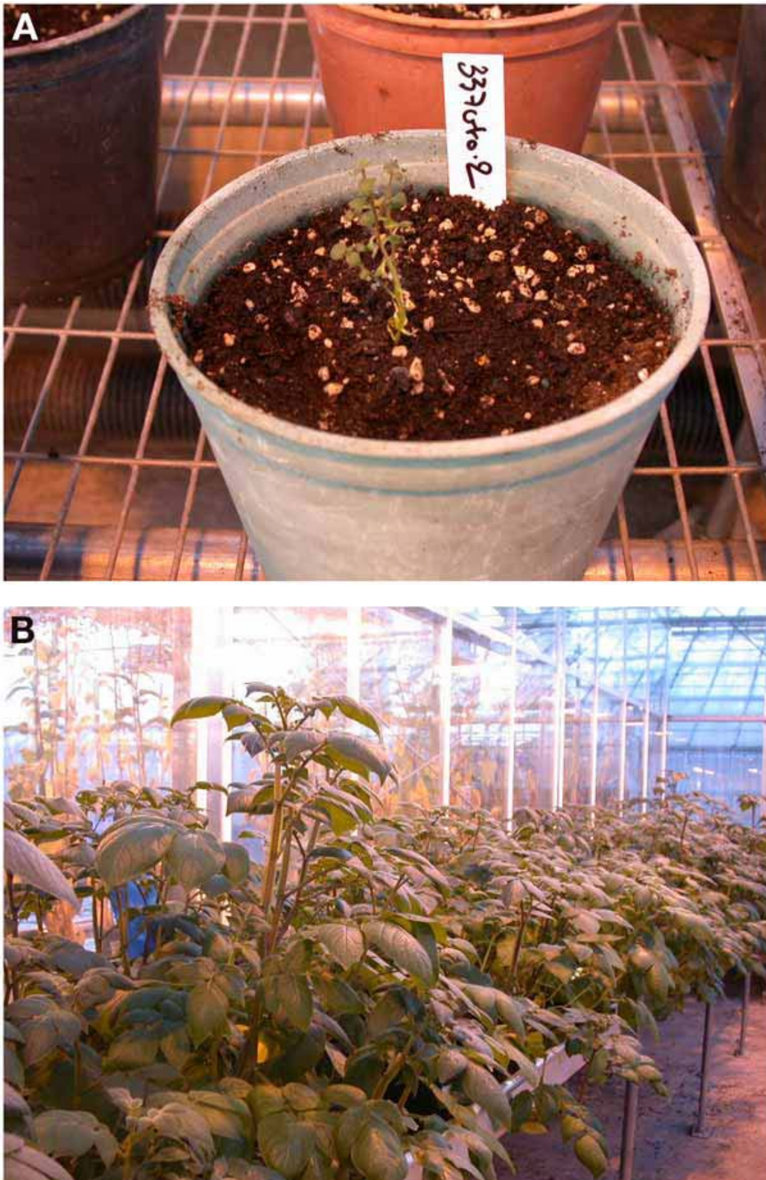

Fig. 2. (A) Newly transplanted shoot in a 12.5-cm pot of compost. (**B)** View of a transgenic population of potatoes in a containment glasshouse, 6 wk post-transplanting.

8. The OD of *Agrobacterium* suspensions is often ignored in many protocols. The optimum will vary according to construct and growth medium, but figures of 0.5–0.8 are generally quoted.

9. The regeneration protocol is also applicable for 5-mm leaf strip sections, and excised 5-mm petiole sections. Regeneration rates vary between cultivars but internodes are often the most responsive and the easiest system for high throughput systems. However, care must be taken in handling to avoid damage, and internodal width of less than 2.5 mm should be avoided.
10. In some protocols which have been shown to be effective in our hands for certain cultivars, an overnight incubation of the explants in a high hormone medium can be employed at this stage. This is formulated as MS20 plus 10X the growth regulators used in CM, and plates should be sealed, and kept overnight in the dark at 18–22°C prior to inoculation with *Agrobacterium* the following day and following the protocol as described.
11. Inoculation methods vary between reports. In some cases a heavy inoculum of *Agrobacterium* is used for a reduced co-cultivation period. In research using *S. phureja* the time period was reduced to 10 min resulting from problems with bacterial overgrowth *(5)*.
12. Inactivation methods will vary from lab to lab and country to country according to local GM regulations. Suitable methods of inactivation may include autoclaving, treatment with bleach, or quaternary detergents.
13. Co-cultivation periods vary between reports. In some cases only 24 h is used, and in others up to 96 h. However, using the system described above, 48 h is optimum. A faint halo of *Agrobacterium* can be seen round the explants at this stage. In the case of overgrowth, washing the explants in 25 mL of 250 mg/L cefotaxime or carbenicillin in MS20 for 60 min in sealed 90-mm Petri dishes on an orbital shaker at 50 rpm prior to plating should alleviate this problem.
14. At this stage it is important not to subculture an excessive number of explants onto the plates of regeneration medium. Reports vary, but figures between 6 and 10 explants per 90-mm plate would appear optimum.
15. This protocol differs from others in that we delay applying selection until the second regeneration medium. In some reports selection is applied directly after the co-cultivation period, or 5 d post co-cultivation. In extensive experiments we have found little advantage in applying immediate selection, in fact regeneration rates can be reduced.
16. Antibiotics and certain growth regulators such as zeatin have been previously shown to break down in the light and thus subculturing at 14-d intervals ensures that the selection pressure is maintained and that levels of antibiotics are high enough to control any risk of *Agrobacterium* overgrowth.
17. If shoot regeneration rates are sufficiently high enough from explants, then a potential problem will be ensuring that any shoots taken for selection are truly independent transgenics, and this can be alleviated by only taking one shoot per explant. However, a possibility may be that the first shoots arising from the explants are escapes, and thus a careful cataloging system must be employed (by numbering each plate, each explant and sequentially numbering shoots taken from each numbered explant) to ensure that, even if several shoots per explant survive selection, only one independent is taken forward for further analysis.

18. Visual selection of putative transgenics is relatively easy in potato as an excellent indicator of the transformed status is the ability to form roots direct from the cut end of the stem. This must not be confused with adventitious rooting from the stem above the cut end. Often the cut end exhibits a curling away from the surface of the medium if it is not transformed. In our hands, two rounds of selection enable more than 95% success rate (confirmed by Southern blotting) of transformation.
19. Glasshouse containment levels vary according to local and national GM Containment regulations and according to the risk of the experiments. A low level of containment is needed for basic transformation experiments and only if the gene products affect pathogenicity or are likely to impact on either environment or human health do more specialized containment facilities become necessary.
20. Variation in transgene expression among the progeny has been reported (*2,17*) and this has been attributed to the random integration of the transgene into different sites of the plant genome. A detailed analysis of significant populations of transgenic lines of potato has shown a number of phenotypic changes and substantially reduced tuber yields in field trials. Such changes have been attributed to epigenetic and genetic events occurring during the regeneration phase of transformation. The frequency of these off-types has been recorded as 15–80%, depending on the potato cultivar (*18*), and often do not become apparent until plants are grown in the field (*19*). Clearly, initial data derived from glasshouse trials or first generation tubers derived from microplants needs to be treated with caution and more emphasis made on results obtained from plants generated through a tuber generation.

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4

Sweet Potato [*Ipomoea batatas* (L.) Lam.]

Guo-qing Song and Ken-ichi Yamaguchi

Summary

Among the available transformation methods reported on sweet potato, *Agrobacterium tumefaciens*–mediated transformation is more successful and desirable. Stem explants have shown to be ideal for the transformation of sweet potato because of their ready availability as explants, the simple transformation process, and high-frequency-regeneration via somatic embryogenesis. Under the two-step kanamycin-hygromycin selection method and using the appropriate explants type (stem explants), the efficiency of transformation can be considerably improved in cv. Beniazuma. The high efficiency in the transformation of stem explants suggests that the transformation protocol described in this chapter warrants testing for routine stable transformation of diverse varieties of sweet potato.

Key Words: *Agrobacterium tumefaciens*; embryogenesis; GUS; *Ipomoea batatas*; stem explants; sweet potato; transformation; transgenic plants.

1. Introduction

Sweet potato [*Ipomoea batatas* (L.) Lam.] is an attractive target for "plant molecular farming" because of its clonal propagation, ease of cultivation, and high productivity of storage roots and foliage. Likewise, male sterility, incompatibility, and a hexaploid genome make it difficult to improve by conventional breeding ***(1)***. Thus, a great deal of effort has been made to establish an efficient transformation system for sweet potato ***(2–11)***. Both particle bombardment and electroporation have been reported to have potential for generating transgenic sweet potatoes ***(2–4)***, although no transformed plants have yet been obtained. Morphologically aberrant transgenic sweet potatoes were regenerated from hairy roots induced on inoculated leaf disks mediated by *Agrobacterium rhizogenes* ***(5)***. Successful recovery of morphologically normal transgenic sweet potatoes has been reported at low efficiency using freshly harvested storage root disks ***(6)***, leaf explants ***(7)*** and embryogenic calli ***(8–11)*** as explants following transformation

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with *A. tumefaciens*. Readily available explant source, effective selection, and efficient regeneration are all desirable for an efficient *A. tumefaciens*–mediated transformation system.

Using a commercially important sweet potato cultivar—Beniazuma—in Japan, we have developed an efficient, somatic embryogenesis-based plant regeneration system for stem explants. A two-step kanamycin-hygromycin selection method has been found to be very effective in selecting transformed plants from inoculated stem explants ***(12)***. Under the optimized transformation conditions, stem explants from the shoots induced from storage roots planted in soil are inoculated and co-cultivated with *A. tumefaciens* strain EHA105 harboring pIG121Hm. The binary vector pIG121Hm contains the *npt*II (p*nos*) gene for kanamycin (Km) resistantce, the *hpt* (p35S) gene for hygromycin (Hyg) resistance, and the *gusA* (p35S) reporter gene for the β-glucuronidase (GUS) ***(13)***. After co-cultivation, induction and selection of transgenic embryogenic calli from the stem explants begins first with culture on 50 mg/L of Km and then transfers to 30 mg/L of Hyg on callus induction medium. Friable embryogenic calluses (FECs) regenerate into plants via somatic embryo on regeneration medium. This transformation protocol has led to successful recovery of transgenic plants shoots from stem explants at 26.3% transformation efficiency defined as percentage of inoculated explants producing β-glucuronidase (GUS) and Polymerase Chain reaction (PCR) positive plants ***(12)***. In addition, one-step selection with Km or Hyg has also resulted in successful regeneration of transgenic plants from stem explants. The protocol described in this chapter can be extended to some of the other sweet potato cultivars.

2. Materials

2.1. Plant Material

Starting plant materials: Storage roots of sweet potato cv. Beniazuma obtained from the National Agriculutural Research Center for Kyushu Okinawa Region, National Agricultural Research Organization (KONARK/NARO, Miyakonojo, Miyazaki, Japan).

2.2. Culture Medium and Stock Solutions

All media are pH adjusted with KOH before adding agar and autoclaving at 121°C for 15 min under 105 kPa. Double distilled water (ddH$_2$O) is used for medium and solution preparation. Antibiotics such as kanamycin and cefotaxime and acetosyringone solution are added to autoclaved media that are cooled down to about 60°C.

1. Linsmaier and Skoog medium (LS) ***(14)***: 1X LS salts and vitamins; 3% (w/v) sucrose, pH 5.6; 0.32% (w/v) gellan gum (Wako Pure Chemical Industries, Ltd., Chuo-ku, Osaka, Japan).

2. 100X stock solutions of LS salts and vitamins (1L): The stocks are autoclaved and stored at 4°C.
   a. LS1: 190 g KNO_3.
   b. LS2: 165 g NH_3NO_3.
   c. LS3: 44 g $CaCl_2 \cdot 2H_2O$.
   d. LS4: 37 g $MgSO_4 \cdot 7H_2O$.
   e. LS5: 17 g KH_2PO_4.
   f. LS6: 4.21 g NaFe·ethylene diamine tetra acetic acid (EDTA).
   g. LS7: 2.23 g $MnSO_4 \cdot 4H_2O$, 0.86 g $ZnSO_4 \cdot 7H_2O$, 0.62 g H_3BO_3, 0.083 g KI, 0.025 g $Na_2MnO_4 \cdot 2H_2O$, 0.0025 g $CoCl \cdot 6H_2O$, and 0.0025 g $CuSO_4 \cdot 5H_2O$.
   h. LS8: 10 g myoinositol and 0.04 g thiamine.
3. Embryogenic callus induction medium (ECIM) ***(15)***: 1X LS salts and vitamins; 1.0 mg/L 4-fluorophenoxyacetic acid (4-FA); 3% (w/v) sucrose, pH 5.6; and 0.32% (w/v) gellan gum (Wako) (for solidified medium). Agar medium is poured into Petri dishes (90 × 20 mm) after adding cefotaxime (25 mL/dish).
4. Regeneration medium (RM) ***(15)***: 1X LS salts and vitamins; 4.0 mg/L abscisic acid (ABA) and 1 mg/L gibberellic acid (GA_3); 3% sucrose, pH 5.6; and 0.32%(w/v) gellan gum (Wako) (for solidified medium). Agar medium is poured into Petri dishes (90 × 20 mm) after adding cefotaxime (25 mL/dish).
5. Growth medium (GM): 1X LS salts and vitamins; 3% sucrose, pH 5.6; and 0.32%(w/v) gellan gum (Wako) (for solidified medium). Add 0.5 mg/L indole-3-acetic acid (IAA), 250 mg/L cefotaxime after autoclave. Agar medium is poured into dishes (25 mL/dish) and GA7 Magenta boxes (50 mL/box).
6. LB medium: 5 g/L Bacto-yeast extract, 10 g/L Bacto-tryptone, 10 g/L NaCl, 18 g/L Bacto™ agar (Becton Dickinson and Company, Sparks, MD) for solidified medium, pH 7.0. Agar medium is poured into Petri dishes (100 × 15 mm) (25 mL/dish).
7. Kanamycin monosulfate (Km) (ICN Biomedicals Inc., Aurora, OH): 50 mg/mL stock in ddH_2O. Sterilize by filtration through 0.22 µm membrane, and store in aliquots at 20°C.
8. Cefotaxime (Wako): 250 mg/mL in ddH_2O. Sterilize by filtration through 0.22 µm membrane, and store in aliquots at 20°C.
9. Hygromycin B (Hyg) solution: 50 mg/mL (Wako).
10. Acetosyringone: 100 m*M* stock. Prepared in dimethyl sulfoxide (DMSO) and stored in aliquots at 4°C.
11. ABA (Wako): 1 mg/mL stock solution. Prepared by dissolving the powder in a few drops of 1*N* KOH and adding dd H_2O to volume. Store at 4°C in the dark.
12. GA_3 (Wako): 1 mg/mL stock solution. Prepared by dissolving the powder in a few drops of 1*N* KOH and adding dd H_2O to volume. Store at 4°C in the dark.
13. 4-FA (Sigma-Aldrich): 1 mg/mL stock solution. Prepared by dissolving the powder in a few drops of 1*N* KOH and adding dd H_2O to volume. Store at 4°C.

2.3. Bacterial Strains and Binary Vector

1. Disarmed *A. tumefaciens* strains EHA105 ***(16)***: Containing the binary vector pIG121Hm. EHA105 culture is suspended in 20% sterile glycerol (v/v), stored in aliquots at −80°C.

2. Binary vector pIG121Hm: A plasmid contains the neomycin phosphotransferase-II gene (*npt*II) for Km resistance under the NOS promoter, the hygromycin phosphotransferase gene (*hpt*) for Hyg resistance under the CaMV 35S promoter, and a plant intron interrupted β-GUS gene (*gus*A) driven by the CaMV 35S promoter ***(13)***.

2.4. Other Solutions and Supplies

1. Sterilizing solution: 5% NaClO (v/v) + 0.01% (v/v) Tween-20.
2. Planting medium: A 2:1 (v/v) vermiculite and perlite mixture.

3. Methods

3.1. Preparation of Stem Explants

1. Pot storage roots of cv. Beniazuma in sterilized planting soil, culture in a growth chamber at 29°C under a 12-h photoperiod of 30 µmol/m^2/s and water the storage roots at 5-d intervals. After 3–4 wk many shoots are induced.
2. Harvest the shoots (10–30 cm in length) from the storage roots, remove the leaves, and cut the shoots into stem segments (7–8 cm in length) (*see* **Fig. 1A**).
3. Wash the segments in flowing tap water for 15 min.
4. Transfer the segments to a 50-mL Corning tube, fill the tube with sterilizing solution, tighten the cap, and shake the tube for 15 min on a rotary shaker at 100 rpm.
5. Pour off the sterilizing solution and wash the stem segments three times (5 min/time) by shaking in sterile water.
6. Remove a 0.5-cm long section from both ends of each stem segment, cut the remaining part transversely into sections that are 6–l0 mm in length. Each section is cut in half along the axis and used as explants for transformation (*see* **Note 1**).
7. Transfer the freshly prepared stem explants onto a sheet of liquid ECIM-soaked sterile filter paper in a dish.

3.2. Infection and Co-cultivation

1. Streak the *A. tumefaciens* strain EHA105: pIG121Hm stock culture onto a LB + 50 mg/L Km plate.
2. Culture the plate for 3 d in an incubator at 30°C.
3. Culture a single colony of the strain EHA105: pIG121Hm in 2 mL of LB + 50 mg/L Km at 30°C for 48 h. Inoculate 20 (L of the culture into 20 mL of the same medium in a 50 mL Corning tube and grow 6–12 h to an OD_{600} of 0.8–1.0.
4. Transfer the culture to 2-mL sterile tubes, centrifuge at 2500*g* for 2 min.
5. Discard the supernatant, suspend the pellets in equal volume of liquid ECIM + 100 µ*M* acetosyringone, and incubate for 1 h at 30°C.
6. Transfer the freshly prepared stem explants to a 50-mL Corning tube, add the bacterial suspension, and then incubate with rotary shaking at 100 rpm for 20 min at 30°C.
7. Pour off the suspension culture and then transfer the stem explants onto two sheets of sterile filter paper in a dish.
8. After blotting dry on sterile filter paper, place the stem explants (20 explants/dish) on sterilized filter paper overlaid on 30 mL of solid ECIM + 100 µ*M* AS in each

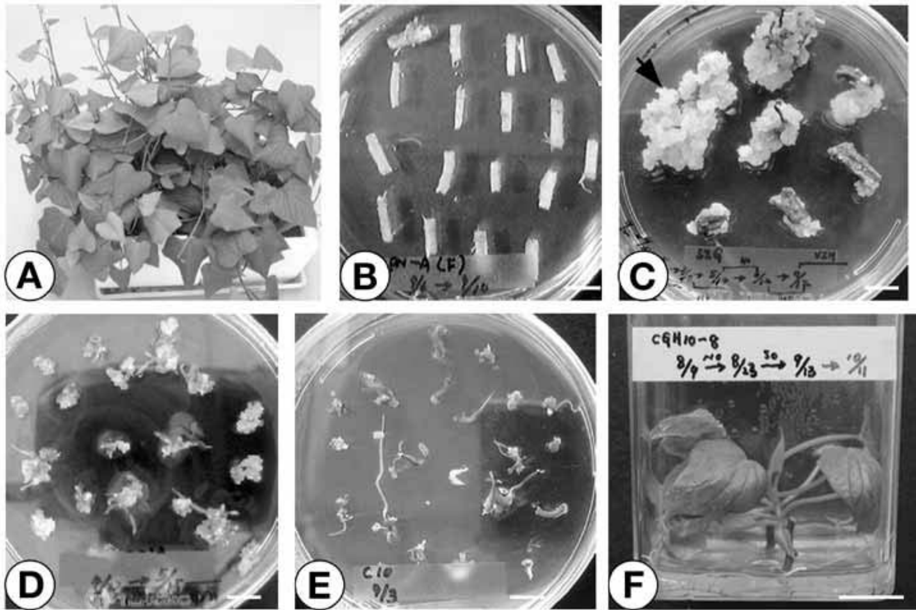

Fig. 1. Transformation of sweet potato using stem explants. (**A**) Shoots induced from a storage root. (**B**) Stem explants transferred onto selection medium after 3-d co-cultivation. (**C**) Formation of hygromycin resistant embryogenic callus cluster after 8 wk of selection on ECIM (arrow indicates an explants that have embryogenic callus clusters). (**D**) Plant regeneration via somatic embryogesis after 4 wk of culture on RM. (**E**) Plantlets on GM. (**F**) Proliferation of transgenic plants by cultivating node sections. *Bars:* 1 cm.

Petri dish. Co-cultivate the explants with EHA105 for 3 d at 23°C in the dark. The dishes are sealed using Parafilm unless otherwise mentioned.

3.3. Selection and Regeneration

1. Transfer the inoculated stem explants into a 50-mL Corning tube.
2. Add 50-mL liquid ECIM supplemented with 500 mg/L cefotaxime in the tube, wash the explants for 10 min with constant shaking by hand.
3. Rinse the explants three times (2 min/time) in 50 mL liquid ECIM without cefotaxime.
4. Transfer the explants into a Petri dish, and blot dry the explants on sterile filter paper.
5. Place the explants on ECIM + 50 mg/L Km + 250 mg/L cefotaxime, keep the cut surface along the axis down in contact with the medium (*see* **Fig. 1B**). Culture for 3 wk at 23°C in the dark (*see* **Note 2**).
6. Transfer the explants onto fresh ECIM + 50 mg/L Km + 250 mg/L cefotaxime. Keep the cut surface along the axis up. Culture for 3 wk at 23°C in the dark.
7. Transfer the explants onto ECIM + 30 mg/L Hyg + 250 mg/L cefotaxime, keep the surface that has new formed calluses in contact with medium; culture at 26°C in the dark.

8. Subculture the explants on fresh ECIM + 30 mg/L Hyg + 250 mg/L cefotaxime at 3 wk intervals at 26°C in the dark.
9. After 3 subcultures on ECIM + 30 mg/L Hyg + 250 mg/L cefotaxime, transfer and culture the stem explants that have resistant embryogenic calli (*see* **Fig. 1C**) on RM + 100 mg/L cefotaxime at 26°C under a 16-h photoperiod of 30 μE/m^2/s. Plant regeneration via somatic embryogenesis occurs from the embryogenic callus clusters in 4 wk (*see* **Fig. 1D** and **Note 3**).
10. Transfer the plantlets derived from individual stem explants onto 30 mL of GM in a petri dish (90 × 20 mm) separately, culture for 2 wk at 26°C under a 16-h photoperiod of 30 μE/m^2/s (*see* **Fig. 1E**).
11. Excise a piece of leaf tissue from morphologically normal plantlets for GUS staining, transfer the remaining, 3–5 plantlets/per Magenta box, onto GM in GA7 Magenta boxes. Culture the plantlets for 2 wk at 26°C under a 16-h photoperiod of 30 μE/m^2/s. Ensure that all plantlets derived from an individual stem explants are cultured separately and treated as an individual transgenic event (*see* **Note 4**).
12. Before transplanting into soil, the morphologically normal plants that have GUS-positive tissues are proliferated in vitro by cultivating node sections on GM without cefotaxime in GA7 Magenta boxes (*see* **Fig. 1F** and **Note 5**).

3.4. Transplanting

1. Wash the in vitro–cultured plantlets in tap water to remove agar around the roots.
2. The plants are transferred to water-soaked planting medium in plastic pots (20 × 30 cm), and cultured in a growth chamber at 25°C under a 16-h photoperiod of 50 μE/m^2/s.
3. Water the plants twice a week. Leaves from these plants can be used for DNA extract or GUS staining (*see* **Note 6**).
4. Propagation of the transgenic plants is carried out by cutting the internode section.

4. Notes

1. After surface sterilization, the stem explants are sterile, and the outer layer cells of stem explants are partly wounded. The surface-wounded stem explants are helpful for subsequent transformation and regeneration because the vascular cells of stems are active in dividing and callusing. When stem explants harvested from in vitro-cultured plantlets are used, it is better to wound the surface of the stem explants before transformation.
2. The two-step Km-Hyg selection method has led an effective selection and efficient regeneration of transgenic plants from stem explants, leaf disks and petioles ***(12)***. However, one selectable marker like the *npt*II or the *hpt* is usually included in most of binary vectors used for transformation. Selection with kanamycin ***(4,6–8)*** or hygromycin ***(9–11)***, transgenic sweet potato plants have been obtained from three cultivars. Thus, we have attempted at one-step selection method using kanamycin or hygromycin independently to select transgenic plants of cv. Beniazuma. The minor modifications of the selection protocol have also led to a successful transformation (*see* **Table 1**).

Table 1
Modification of the Selection and Regeneration Media and Protocols When the *npt*II or the *hpt* is the Only Selectable Marker Gene in the Vector

Selectable marker gene		Selection and regeneration	Transformation frequency (%)
*npt*II	1.	After co-cultivation, explants were placed on ECIM + 100 mg/L Km + 250 mg/L cefotaxime to select the transformed cells.	
	2.	Regeneration from resistant embryogenic calluses was performed on RM + 100 mg/L Km + 250 mg/L cefotaxime.	19.6
hpt	1.	After co-cultivation, culture stem explants on ECIM + 250 mg/L cefotaxime for 6 wk, then select the transformed cells on ECIM + 30 mg/L Hyg + 250 mg/L cefotaxime.	
	2.	RM + 100 mg/L cefotaxime was used to regenerate plants from resistant embryogenic calluses.	12.7

3. Not all resistant calli obtained are embryogenic. Only embryogenic calli that are bright-yellow and friable calluses can regenerate via somatic embryogenesis on RM. Embryogenic calli can be maintained and proliferated on ECIM for 2 mons. Over 2 mons, a loss of embryogenic characteristics will happen in some callus clusters. We usually transfer half of the embryogenic calli from each explants onto RM, and the remaining are maintained on ECIM for a backup.
4. Tens to hundreds of plantlets can be obtained from each independent transgenic event because of the plant regeneration via somatic embryogenesis. About 5–15% of the regenerants usually have phenotypic abnormalities such as showing reduced apical dominance and/or a reduced internode length, and a failure to produce roots and recover a normal phenotype. We usually pick up between 5 and 10 morphologically normal plants from each transgenic event for further analysis.
5. Using this method, an individual transgenic plant can be proliferated quickly.
6. When a *gus*A interrupted by a plant intron is used, GUS staining is a reliable option to detect transformed plant tissues; otherwise, plant tissues from soil grown plants are preferable for histochemical GUS assay. Stable integration and the number of T-DNA inserts should be confirmed using Southern blot assay.

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II

Turf Grasses

5

Bermudagrass (*Cynodon* spp.)

Yaxin Ge and Zeng-Yu Wang

Summary

Bermudagrass is an important warm-season forage and turf species widely grown in the southern United States. This chapter describes a rapid and efficient protocol that allows for the generation of a large number of transgenic bermudagrass plants, bypassing the callus formation phase. Stolon nodes are infected and co-cultivated with *Agrobacterium tumefaciens* harboring pCAMBIA binary vectors. Hygromycin phosphotransferase gene (*hph*) is used as the selectable marker and hygromycin is used as the selection agent. Green shoots are directly produced from infected stolon nodes 4 to 5 wk after hygromycin selection. Without callus formation and with minimum tissue culture, this procedure allowed us to obtain well-rooted transgenic plantlets in only 7 wk and greenhouse-grown plants in only 9 wk.

Key Words: *Agrobacterium*; bermudagrass; *Cynodon*; forage and turf grass; transformation; transgenic plants.

1. Introduction

Bermudagrass (*Cynodon* spp.) is a warm-season C4 perennial species widely used for forage and turf purposes. It is found in more than 100 counties throughout the tropical and subtropical areas of the world. In the United States, the distribution of bermudagrass extends from New Jersey and Maryland southward to Florida and westward to Kansas and Texas. Bermudagrass is a deep-rooted, sod-forming grass that spreads by means of stolons, rhizomes, and seed. Although both seeded and sprigged varieties of bermudagrass are available, sprigged varieties generally have a yield advantage over seeded varieties. In commercial production, more bermudagrass is propagated by planting sprigs than by seeding; farmers have generally adopted the sprig method. Poor seeding habits of some improved cultivars make it mandatory that they be established from vegetative material *(1)*. This offers a significant advantage for

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biotechnological improvement of bermudagrass, because a transgenic line with desired agronomic traits has the potential to be directly propagated and used as a cultivar for commercial purposes. This will avoid problems with seed production (e.g., crossing, segregation, level of expression in the progenies) and allow a much quicker release of cultivars. Furthermore, the use of triploid sterile hybrid cultivars could eliminate the pollen or seed-mediated transgene flow problem occurring in most other transgenic plants *(2)*.

Transgenic bermudagrass has only been reported by biolistic transformation of embryogenic calluses *(2–4)*. In this protocol, we describe the generation of transgenic bermudagrass by *Agrobacterium*-mediated transformation. It is known that *Agrobacterium*-mediated transformation has the advantage of allowing for the stable integration of a defined DNA segment into the plant genome and generally results in a lower copy number, fewer rearrangements and an improved stability of expression over generations than the free DNA delivery methods *(5,6)*.

Callus culture has been an inevitable step in grass transformation. It is well known that callus induction and plant regeneration from the induced callus is not only time consuming and laborious, but also causes somaclonal variation (*3,7,8*). The protocol described here bypasses the callus formation phase and allows for direct regeneration of transgenic plants from *Agrobacterium*-infected stolon nodes (*9*). The protocol allows for rapid and efficient production of transgenic bermudagrass (*see* **Note 1**). Based on the number of transgenic plants obtained and the number of stolon nodes inoculated, transformation efficiency of 6.1% was achieved (*see* **Note 2**).

2. Materials

2.1. Plant Material

Plants of triploid bermudagrass (*Cynodon dactylon* × *C. transvaalensis*) cultivar, TifEagle *(10)*, are grown in the greenhouse (390 μE/m^2/s, 16-h d/8-h night at 24°C/20°C) (*see* **Note 3**).

2.2. Agrobacterium tumefaciens *Strain and Selectable Marker*

The *Agrobacterium tumefaciens* strain EHA105 is used in combination with the binary vector pCAMBIA1301, which carries a hygromycin phosphotransferase gene (*hph*) and a β-glucuronidase (GUS) gene (*gusA*), both under the control of CaMV 35S promoter (*www.cambia.org*). Hygromycin is used as selection agent.

2.3. Culture Media for Agrobacterium tumefaciens

1. Luria Broth (LB) medium: LB-Agar capsules (Qbiogene, Montreal, Canada).

2. AB medium (*11*): The AB medium (per L) contains 3000 mg K_2HPO_4, 1000 mg NaH_2PO_4, 1000 mg NH_4Cl, 300 mg $MgSO_4{\cdot}7H_2O$, 150 mg KCl, 10 mg $CaCl_2{\cdot}2H_2O$, 2.5 mg $FeSO_4{\cdot}7H_2O$, 5000 mg glucose. Autoclave.

2.4. Tissue Culture and Establishment of Soil-Grown Plants

1. Commercial bleach.
2. 2,4-Dichlorophenoxy-acetic acid (2,4-D): Prepare 100 mg/L stock by dissolving the powdered chemical in a few drops of 1*M* KOH and add double distilled water (ddH_2O) to volume.
3. Kinetin: 1 mg/mL stock (PhytoTechnology Laboratories, Shawnee Mission, KS).
4. BG-A1 medium: Half-strength Murashige and Skoog (MS) medium (Phyto Technology Laboratories) supplemented with 4.5 µ*M* kinetin, 1.8 µ*M* 2,4-D, 3.3 m*M* L-cysteine, 1 m*M* dithiothreitol, 1 m*M* Na-thiosulfate and 2% (w/v) sucrose. Adjust pH to 5.8 with KOH. For liquid medium, filter-sterilize the solution. For solid medium, add 8 g agar and autoclave.
5. Acetosyringone (ACROS Organics, Morris Plains, NJ): Prepare fresh 100 m*M* acetosyringone (3′,5′-Dimethoxy-4′-hydroxyactophenone) by dissolving acetosyringone in dimethyl sulfoxide (DMSO).
6. Hygromycin: 100 mg/mL stock (PhytoTechnology Laboratories).
7. Cefotaxime (Agri-Bio, North Miami, FL): Prepare 250 mg/mL stock in ddH_2O, filter-sterilize and store at −20°C.
8. Tobacco juice: Freshly prepared by squeezing sterile leaves with a spatula.
9. BG-A2 medium: MS medium (PhytoTechnology Laboratories) supplemented with 4.5 µ*M* kinetin, 0.2 µ*M* 2,4-D, 2% sucrose and 0.8% agar. Adjust pH to 5.8 with KOH, autoclave and then add 75 mg/L hygromycin, 1.7 mg/L silver nitrate, and 250 mg/L cefotaxime (*see* **Note 4**).
10. MSO medium: half-strength MS medium (PhytoTechnology Laboratories) solidified with 0.8% (w/v) agar. Adjust pH to 5.8 and autoclave.
11. Sterile distilled water.
12. Sterile 6.6-cm diameter (190 mL) plastic culture vessels (Greiner Bio-One, Longwood, FL).
13. Sterile 5.0-cm diameter (175 mL) plastic culture vessels (Greiner Bio-One, Longwood, FL).
14. Sterile plastic Petri dishes, Parafilm.
15. Forceps, scalpel and blades
16. Autoclaved glass flask (125 mL).
17. Drummond Pipet-Aid and sterile disposable pipettes.
18. Magnetic stirrer and stir bars.
19. Shaker/incubator.
20. A rotary shaker.
21. Nalgene transparent polycarbonate desiccator.
22. A swing rotor centrifuge.
23. Spectrophotometer.
24. Metro Mix 350 soil (Sun Gro Horticulture, Terrell, TX).

3. Methods

3.1. Agrobacterium Preparation

1. Streak *A. tumefaciens* from a glycerol stock onto LB agar plate with antibiotic selection appropriate for the vector used. Incubate at 28°C for 2 d.
2. Transfer a single colony from the plate into a flask containing 60 mL AB medium with antibiotic selection appropriate for the vector used.
3. Incubate the cultures on a shaker/incubator at 200 rpm at 28°C for about 2 d, until the culture has reached an OD_{600} of 1.0. Add 50 µL 100 m*M* acetosyringone and continue shaking for 2 more h.

3.2. Sterilization and Preparation of Explants

1. Collect stolons from the greenhouse-grown plants, tear off leaves and cut into 2- to 3-cm segments with nodes in the middle of the segments (*see* **Fig. 1A**).
2. Sterilize the segments in 70% ethanol for 1 min and 20% commercial bleach (with a few drops of Tween-20) for 10 min and then rinse three times with sterile water (*see* **Note 5**).
3. Cut the nodes in half and place them in culture vessels. The small segments (0.5 to 1.0 cm), each containing a cut node, are ready for *Agrobacterium* infection (*see* **Fig. 1B**).

3.3. Inoculation of Explants and Cocultivation

1. Centrifuge the *Agrobacterium* culture at 2400*g* for 15 min.
2. Pour off supernatant, resuspend the pellet with liquid BG-A1 medium, adjust OD_{600} to about 1.0.
3. Add 30 mL *Agrobacterium* suspensions to the stolon nodes in the culture vessels; add 0.5 mL tobacco juice (*see* **Note 6**).
4. Place the culture vessels in a polycarbonate desiccator and draw vacuum (62 cm Hg) for 10 min.
5. Release vacuum, incubate the stolon nodes and *Agrobacterium* for 50 min with gentle shaking on a rotary shaker.
6. Remove the bacteria after the incubation, transfer the stolon nodes onto solidified BG-A1 medium and placed them in the dark at 25°C for co-cultivation.

3.4. Selection and Direct Plant Regeneration

1. After 2 d of co-cultivation, transfer the infected stolon nodes onto BG-A2 selection medium. Place the cultures at 25°C in fluorescent light (40 $\mu E/m^2/s$) at a photoperiod of 16 h in a growth chamber (*see* **Fig. 1C**).
2. After 1 wk, transfer the stolon nodes to new BG-A2 medium (*see* **Fig. 1D**) and keep at the same condition (*see* **Note 7**).
3. Hygromycin-resistant green shoots could be obtained after three to four weeks of selection (*see* **Fig. 1D,E**). Nonresistant shoots turn albino (*see* **Fig. 1F**).
4. After 4 to 5 wk of selection, transfer the green shoots to 5.0-cm culture vessels containing MSO medium (*see* **Fig. 1G**).

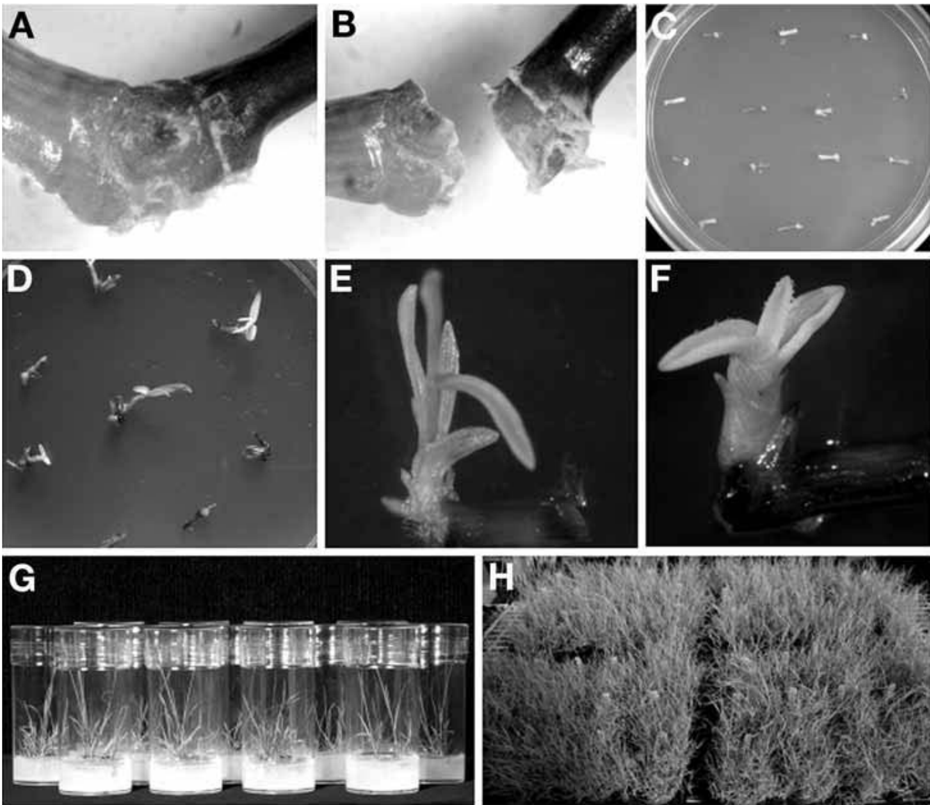

Fig. 1. Rapid production of transgenic bermudagrass plants from stolon nodes after *Agrobacterium tumefaciens*-mediated transformation. (**A**) Stolon segment used for sterilization. (**B**) A cut was made at the node of the stolon segment. (**C**) Infected stolon nodes placed on selection medium. (**D–F**) Green and albino shoots of bermudagrass produced 3 to 4 wk after hygromycin selection. (**G**) Rooted transgenic plantlets obtained 7 wk after transformation. (**H**) Greenhouse-grown transgenic bermudagrass plants.

3.5. Greenhouse Care and Propagation

1. Transfer well-rooted plantlets (*see* **Fig. 1G**) to 3 × 3 inch wells in an 18-well flat (6 × 3 wells) filled with Metro Mix 350 soil (*see* **Note 8**) and grow them under greenhouse conditions (390 µE/m^2/s, 16-h d/8-h night at 24°C/20°C). Plants can be grown on Ebb-Flo® benches and watered once a day with fertilized water containing 50 ppm N (Peters Professional 20-10-20 General Purpose is used as the water soluble fertilizer).
2. Transfer the established plants to 4.5 inch pots filled with Metro Mix 350 soil and grow them under greenhouse conditions (390 µE/m^2/s, 16-h d/8-h night at 24°C/20°C) (*see* **Fig. 1H**).

3. Because the cultivar used is sterile, the transgenic material can be propagated by cutting and replanting the stolons to new pots.

4. Notes

1. We have also successfully produced transgenic creeping bentgrass and zoysiagrass plants using a similar protocol. The transformation efficiency in creeping bentgrass was up to 11.3%.
2. When callus is used for transformation, the final transformation efficiency depends on the frequency of embryogenic callus formation, the percentage of resistant calluses obtained after antibiotic selection and the frequency of plant regeneration from the resistant calluses. Transformation frequency is often reported as the number of transgenics divided by the number of calluses used in the literature. If the frequency of embryogenic callus formation is also taken into account, the callus based transformation efficiency is generally lower than the efficiency reported here.
3. By using this protocol, we have been able to produce transgenic plants from a forage-type bermudagrass cultivar, Midland 99.
4. The hygromycin concentration used for creeping bentgrass selection is 100 mg/L.
5. If the stolons are dirty, the sterilization time can be extended, or sterilize twice (2×6 min). Make sure all stolons have good contact with the solution.
6. The use of tobacco juice is helpful for increasing transformation efficiency.
7. If *Agrobacterium* overgrow, wash the explants with 500 mg/L cefotaxime, dry with sterile filter paper and put back to new selection medium.
8. Before transfering to soil, rinse the roots with water or remove excessive medium with a damp paper towel.

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6

Perennial Ryegrass (*Lolium perenne* L.)

Fredy Altpeter

Summary

A protocol that facilitates rapid establishment of *Agrobacterium*-mediated transformation for perennial ryegrass is described. The synthetic green fluorescent protein (sgfpS65T) reporter gene is introduced in combination with the *npt*II selectable marker gene into axillary bud derived embryogenic calli of perennial ryegrass (*Lolium perenne* L.) by co-cultivation with *Agrobacterium tumefaciens* strain AGL0 harboring binary vector pYF132. Following the co-cultivation calli are cultured for 48 h in liquid callus medium containing timentin at 10°C and 70 rpm, which reduces *Agrobacterium* overgrowth. Using green fluorescent protein (GFP) as a nondestructive visual marker allows identification of responsive genotypes and transgenic cell clusters at an early stage. GFP screening is combined with paromomycin selection to suppress wild type cells. Transgenic plantlets ready to transfer to soil are obtained within 4 mo of explant culture. Between 8 and 16% of the *Agrobacterium*-inoculated calli regenerate independent, Southern positive transgenic plants. Reproducibility and efficiency in this perennial ryegrass transformation protocols is controlled by multiple factors including genotype dependent tissue culture and gene transfer response, a short tissue culture-and-selection period and the efficient suppression of *Agrobacterium* following *Agrobacterium*-mediated gene transfer.

Key Words: Ryegrass; *Lolium perenne* L.; *Agrobacterium*-mediated gene transfer; *npt*II; *gfp*; grass transformation.

1. Introduction

Perennial ryegrass is one of the most widely cultivated grasses in the temperate regions *(1)*. Genetic transformation is a powerful tool for improvement of turf and forage grasses including perennial ryegrass *(2)*. A range of gene transfer protocols have been described for the production of transgenic perennial ryegrass plants including biolistic gene transfer using DNA-coated microprojectiles *(3,4)*, silicon carbide fiber–mediated gene transfer *(5)* or direct gene transfer into protoplast *(6)*. Initial protocols required a long tissue culture period, which is

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more likely to result in undesirable somaclonal variation (*7*). Large numbers of fertile transgenic perennial ryegrass plants were generated with accelerated tissue culture and selection following biolistic gene transfer (*8*).

Compared to *Agrobacterium*, biolistic gene transfer is usually successful in a wider range of genotypes (*8*), and co-transfer of multiple genes is facilitated (*9*). *Agrobacterium*-mediated gene transfer, however, offers potential advantages over biolistic gene transfer. These include preferential integration of T-DNAs into transcriptional active regions (*10,11*) and elimination of selectable marker genes is facilitated by frequent integration of co-transformed T-DNAs into separate chromosomes (*12,13*). Grasses are not among the natural hosts of *Agrobacterium*, but in the last decade convincing molecular evidence of stable *A. tumefaciens*–mediated gene transfer was presented for rice (*14*), wheat (*15*), barley (*16*), sorghum (*17*), bentgrass (*18*), switchgrass (*19*), zoysiagrass (*20*), rye, (*21*), fescue (*22*) and annual ryegrass (*22*). The transfer of T-DNA and its integration into the plant genome is influenced by the vector-plasmid (*23*), the bacteria strain (*12,23*), the addition of *vir*-gene inducing synthetic phenolic compounds (*24*), culture media composition, culture conditions (*25*) and osmotic stress treatments (*26*) during and before *Agrobacterium* infection, the plant genotype, explant, tissue culture protocol, as well as the suppression and elimination of *Agrobacterium* after co-cultivation (*27*).

This chapter describes a detailed protocol for stable *Agrobacterium*-mediated genetic transformation of perennial ryegrass. This genetic transformation protocol has supported the generation of transgenic ryegrass plants with 8 to 16 Southern positive, independent events from 100 inoculated freshly induced callus pieces. Using green fluorescent protein (GFP) as a nondestructive visual marker allows identification of transgenic cell clusters at an early stage, tracking their fate and reducing time in tissue culture. GFP was used earlier as reporter gene for rapid establishment and improvement of biolistic transformation in corn (*28*), rice (*29*), barley (*30*) and wheat (*31*). In contrast to results obtained by biolistic transformation we found a close correlation between *gfp*-expression shortly after *Agrobacterium*-mediated gene transfer and stable transformation events. This indicates that the monitoring of successful gene transfer is a time saving step in this protocol and will facilitate its establishment in a different laboratory.

2. Materials

2.1. Agrobacterium *Strain and Plasmid*

Electroporation (*32*) (*see* also **Chapter 3**, Volume 1) was used to introduce the binary vector pYF132 into *Agrobacterium tumefaciens* strain AGL0 (*33*). pYF132 encodes the selectable marker neomycin phosphotransferase gene (*npt*II) and the sgfpS65T gene both under control of the corn ubiquitin promoter

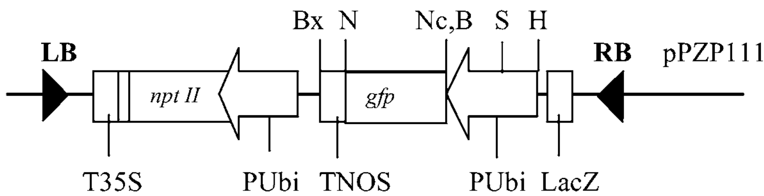

Fig. 1. Diagram of the binary plasmid pYF132 (not drawn to scale). LB, left border; RB, right border; T35S, polyadenylation signal of CaMV35S; P35S, CaMV35S promoter; TNOS, polyadenylation signal of nopaline synthase; PUbi, promoter and first intron of the maize ubiquitin 1 gene; *npt*II, neomycin phosphotransferase; *gfp,* s*gfp*S65T gene; *LacZ,* β-galactosidase complementation factor; Bx, *Bst*XI; *N, Not*I; *Nc, Nco*I; B, *Bam*HI; S, *Sal*I; H, *Hin*dIII; pPZP111, vector backbone pPZP111.

with first intron ***(34)*** and the CaMV 35S and Nos terminator respectively, in the pPZP111 vector-backbone ***(35)*** (*see* **Fig. 1** and **Note 1**).

2.2. Agrobacterium *Culture Media*

1. Luria Broth (LB): 5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl, pH 7.5.
2. Kanamycin monosulfate: 100 mg/mL stock solution in water, filter sterilize (0.2 µm syringe filter) and store 1 mL aliquots at –20°C.
3. Rifampicin: 100 mg/mL stock solution in dimethyl sulfoxide (DMSO), store 1 mL aliquots at –20°C.
4. Liquid culture medium: LB medium with 50 mg/L rifampicin and 50 mg/L kanamycin monosulphate, pH 7.5.
5. Solidified culture medium: LB medium with 10 g/L agar, pH 7.5, with 50 mg/L rifampicin and 50 mg/L kanamycin monosulphate.

2.3. Donor Plant Production to Obtain Tissue Culture Explants

Plants of perennial ryegrass (*Lolium perenne* L.) cultivar Limes (DSV GmbH Lippstadt/Germany) are grown in the greenhouse at 12°C to 16°C during night and 16°C to 20°C during the day with 12/12 h light/dark cycle (*see* **Note 2**). The soil mixture consists a 3:1:1 mixture of topsoil:peat:sand, and plants are fertilized biweekly with Peters-fertilizer (20:20:20 with micronutrients), following the manufacturers recommendations. Light intensity of at least 360 µE/m^2/s at plant height is maintained with sodium vapor lights (Sonagro, Phillips). Tillers are harvested, roots removed and tillers are surface sterilized before excision of axillary buds.

2.4. Perennial Ryegrass Tissue Culture Media

1. Sterilizing solutions: 70% (v/v) ethanol; sodium hypochlorite solution (2.4% active Chlorine) supplemented with a surfactant (0.1% (w/v) of Tween-20).

2. Vitamin stock: Murashige and Skoog (MS) *(36)* Vitamin Mixture 1000X (PhytoTechnology, Shawnee Mission, KS). Dissolve 10.31 g of the premixed vitamins in 100 mL water. Filter sterilize (0.2 µm) and store 1 mL aliquots at –20°C.
3. 2,4-Dichlorophenoxyacetic acid (2,4-D): 2 mg/mL stock solution. Prepare by dissolving in a minimum amount of warm (60°C) 1M KOH and make to volume with water. Store 1 mL aliquots at 4°C.
4. Acetosyringone 100 mM stock solution: Dissolve 196.2 mg of acetosyringone in 10 mL DMSO.
5. Paromomycin sulfate 50 mg/mL stock solution: Dissolve in water, filter sterilize (0.2 µm), and store 1 mL aliquots at –20°C.
6. Timentin 150 mg/mL stock solution: Dissolve in water filter sterilize (0.2 µm) and use immediately.
7. Maltose 10X stock solution: Dissolve 300 g/L maltose in water, pH 5.8, filter sterilize (0.2 µm), and use immediately.
8. Callus induction medium (CIM): 4.3 g basal MS salts *(28)*, 1.5 mL/L 2,4-D stock solution, pH 5.8; 3.0 g/L phytagel, 1 mL/L MS 1000X vitamin stock, 100 mL/L maltose 10X stock solution.
9. Osmotic treatment medium (OTM): CIM plus 72.9 g/L mannitol.
10. Co-cultivation medium, liquid (CCML): 4.3 g basal MS salts, 3.0 mL/L 2,4-D stock solution; 15 g/L glucose; pH 5.2; 2.0 mL/L acetosyringone stock solution (*see* **Notes 3 and 4**), 50 mL/L maltose 10X stock solution.
11. Co-cultivation medium, solid (CCMS): CCML solidified with 3.0 g/L phytagel.
12. Subculture medium, liquid with Timentin (SCML): 4.3 g basal MS salts, 1.5 mL/L 2,4-D stock solution, pH 5.8; 1 mL/L 1000X MS vitamin stock, 1 mL/L timentin stock solution, 100 mL/L maltose 10X stock solution.
13. Callus selection medium (CSM): 4.3 g basal MS salts, 1.5 mL/L 2,4-D stock solution; pH 5.8; 6.0 g/L agarose, 1 mL/L 1000X MS vitamin stock, 1 mL/L timentin stock solution, 1.0 mL/L paromomycin sulphate stock solution (*see* **Note 5**), 100 mL/L maltose 10X stock solution.
14. Shoot and root regeneration and selection medium (SRRSM): 4.3 g basal MS salts, 20 g/L sucrose; pH 5.8; 6.0 g/L agarose; 1 mL/L 1000X MS vitamin stock, 1.0 mL/L timentin stock solution; 1.0 mL/L paromomycin sulphate stock solution (*see* **Note 5**).
15. All media are sterilized by autoclaving at 121°C, 1.5 bar for 15 min. Water was purified with a Milli-Q water purification system. Antibiotics, and vitamins are added to the medium as concentrated, filter-sterilized solutions after autoclaving and at a medium temperature of less than 50°C. Acetosyringone is added after autoclaving. Media containing Timentin are used immediately after preparation, others are stored at room temperature for up to 2 wk.

3. Methods

3.1. Explants and Agrobacterium *Preparation*

1. Surface sterilization: Rinse tillers for 3 min in 70% (v/v) ethanol and for 15 min in 50% sodium hypochlorite solution (1.2% active chlorine) containing approx

0.1% (w/v) of Tween-20 while shaking at 50 rpm, followed by 5 washes with previously autoclaved (121°C, 1.5 bar for 15 min) water.

2. Excise axillary buds approx 4–10 mm in size and place on callus induction medium (CIM). Culture 12 explants per 90-mm Petri-dish at 20 µE/m^2/s and 25°C for 28 to 56 d and subculture to fresh CIM every 14 d.
3. Grow *A. tumefaciens* strain AGL0 harboring vector pYF132 on LB agar culture medium (with antibiotics) at 28°C for 2 d. Transfer one colony of bacteria to 2 mL of LB liquid culture medium (with antibiotics) and grow overnight at 28°C on an orbital shaker at 230 rpm (*see* **Notes 6** and **7**).
4. Measure absorbance at 660 nm of a 1 mL aliquot of the bacterial overnight culture in a spectrophotometer (expected OD_{660} value is 2.0–2.5).
5. Centrifuge 1 mL of the bacterial culture at 13,000*g* for 5 min, discard the supernatant, resuspend the pellet and dilute the suspension to an OD_{660} value of (1.5–2.0) in 1/1 (v/v) LB medium/CCML (without antibiotics) and incubate at 28°C on an orbital shaker at 230 rpm for 2 h before co-cultivation.

3.2. Inoculation, Co-cultivation, Selection, and Regeneration of Transgenic Plants

1. For osmotic treatment place 15–20 embryogenic calli (*see* **Subheading 3.1.2.**) in the center of a 90-mm Petri-dish with OTM medium for 4–6 h prior inoculation with *Agrobacterium*.
2. Inoculate 15–20 embryogenic calli per 90-mm Petri-dish by pipetting approx 200 µL *Agrobacterium* suspension on the calli (*see* **Subheading 3.1.5.**), vacuum treat at 500–800 mbar for 1 min and keep in the laminar flow hood for 10 min.
3. Transfer calli briefly to blotting paper to remove excess *Agrobacterium*, followed by a co-culture on CCMS medium for 44 to 48 h at 22°C and 20 µE/m^2/s.
4. After 44 to 48 h co-cultivation in CCMS medium, rinse explants thoroughly (at least 5 times) in SCML medium and culture approximately 10 calli per 100 mL Erlenmeyer flask with 20 mL SCML medium for 48 h at 10°C and 70 rpm (*see* **Note 7**) in the dark.
5. Blot calli dry on a filter paper and transfer calli to CSM to suppress nontransgenic events. Maintain cultures at 20 µE/m^2/s for 4 wk at 25°C with a subculture to fresh CSM medium after 14 d (*see* **Note 8**).
6. Expression of s*gfp* (S65T) appears as defined fluorescent spots (*see* **Note 8**) under a stereomicroscope equipped with a fluorescent module (*see* **Note 9**). Five to seven days after co-cultivation, the number of GFP expressing signals per callus declines. Approximately 10% of the cells expressing GFP two days after co-cultivation developed into fluorescing calli. Ten to fourteen days after transfer to selection medium green fluorescence of calli under a fluorescent light source and rapid growth of these calli on culture media with paromomycin are co-existing events. In regenerated shoots a strong red autofluorescence from chlorophyll masks the GFP signal. The strong fluorescence in roots is therefore the most reliable indicator for the transgenic character of the regenerated plantlets.
7. Transfer calli to 90-mm Petridish with SRRSM medium and culture at a 16 h photoperiod, 130 µE/m^2/s illumination at 25°C for shoot regeneration for 3 wk (*see* **Note 10**).

8. Transfer 5 regenerating calli to a Magenta box (Sigma-Aldrich) with 40 mL SRRSM medium and culture at a 16 h photoperiod, 130 µE/m^2/s illumination at 25°C for shoot elongation and root formation of transgenic shoots within 3 wk (*see* **Note 10**).
9. It takes approx 7 mo from transplanting until harvest of mature seeds. Rooted transgenic plantlets were transferred to soil after carefully washing off medium from the roots. Topsoil, peat, and sand were mixed in a 3:1:1 ratio for plant growth. To support acclimation plantlets were covered with a Magenta box for the first four days following transfer to soil. Plants are grown in the growth chamber at 12°C to 16°C during night and 16°C to 20°C during the day with 12/12 h light (400 µE/m^2/s) /dark cycle for 3 wk. Then they were vernalized at 4°C 12/12 h light (100 µE/m^2/s) /dark cycle for 10 wk. Following vernalization plants are grown in the growth chamber at 12°C to 16°C during night and 16°C to 20°C during the day with 12/12 h light/dark cycle until end of tillering. After tillering a 16/8 h light/dark cycle is used to produce reproductive tillers. Plants were fertilized biweekly with Peters-fertilizer (20:20:20 with micronutrients; St. Louis, MO), following the manufacturers recommendations. Light intensity of 400 µE/m^2/s at plant height was maintained with metal halide lights until the end of tillering. After tillering sodium vapor lights provided the same light intensity. As soon as immature inflorescenses emerged and before pollination, each transgenic inflorescence is covered together with a wildtype inflorescense with a cellophane bag and pollination within the bags is enhanced with daily agitation of the bags. Under these conditions more than 70% of the transgenic lines were fertile and seed set varied between 10 and 70%.
10. Regenerated plants and their progeny can be assessed for expression of the *npt*II transgene by a commercially available enzyme linked immunosorbent assay (ELISA)-kit (Agdia, Elkhart, IN) (*see* **Note 11**). Southern blot analyses is performed to confirm the stable integration of the transgenes. Northern blot, western blot or ELISA analyses are used to confirm the expression of the GFP or alternative co-transferred transgenes.

4. Notes

1. The binary plasmid used (pYF132) has incorporated bacterial resistance to kanamycin. *A. tumefaciens* strain AGL0 has chromosomal resistance to rifampicin (*33*).
2. Tissue culture response is highly dependent on donor plant quality. Therefore it is important to avoid stresses including drought, temperatures above 25°C, pests, diseases, and pesticides. Ryegrass is a cross-pollinated species, resulting in a significant genotypic variability within a cultivar. This variability reduces also the reproducibility of tissue culture and transformation response from ryegrass cultivars. In the early transformation experiments cross-pollinating ryegrass populations were used or cell suspension derived from a single explant (*3*). The identification of inbred lines displaying a good regeneration response from tissue cultures has also been described (*8*). In contrast to single embryo derived cell suspensions, axillary bud, or immature inflorescense derived callus as target for gene

transfer also allow the introduction of transgenes into an isogenic background but allow avoidance of extended tissue culture periods.

3. Co-cultivation in a medium rich in auxins induces cell division, callus proliferation and maintains tissues in an undifferentiated state, which might enhance transformation competence.
4. The supplementation of CCML and or CCMS medium with Acetosyringone, used prior and during co-cultivation almost doubles the transformation efficiency in perennial ryegrass.
5. Gelling agents may cause precipitation of paromomycin rendering it inactive as a selective agent. Agarose does not cause precipitation of paromomycin.
6. In a series of transformation experiments with the same plasmid, reproducibility can be improved by using 20 μL of an *Agrobacterium* stock stored in glycerol as an inoculum instead of colonies. For the preparation of glycerol stocks a 100 mL *Agrobacterium* suspension can be grown overnight (as a subculture of the culture initiated in 3.1.3 in 100 mL LB medium). After mixing 1:1 with an autoclaved Glycerol solution (30%) the aliquoted suspension can be stored for several months at –80°C.
7. Ryegrass tissue cultures are very sensitive to *Agrobacterium* overgrowth. The described thorough rinsing of the explants for two days on a shaker at 10°C and the use of timentin reduces the potential of *Agrobacterium* overgrowth.
8. The successful *Agrobacterium*-mediated transformation in perennial ryegrass is very genotype dependent. Using GFP as a nondestructive visual marker allows identifying responsive genotypes 2 d after co-cultivation, because there is a good correlation between fluorescent signals shortly after co-cultivation and stable transformation events.
9. The expression of GFP was visualized using a ZEISS stemi SV6 stereomicroscope (Carl Zeiss, Germany) with a 50W mercury lamp, a BP470/20nm excitation filter, and a BP505-530 barrier filter.
10. Paromomycin, compared to other selective agents, is very effective in suppressing elongation of nontransgenic shoots and roots. Effective inhibition of shoot and root growth by paromomycin depends also on the light intensity during regeneration. Under the described selection and culture conditions (130 $\mu E/m^2/s$ illumination) none of the plantlets growing roots inside the 50 mg/L containing paromomycin medium were nontransgenic escapes. In contrast, under 60 $\mu E/m^2/s$ instead of 130 $\mu E/m^2/s$ illumination approximately 30% of the regenerated and root forming plantlets were nontransgenic escapes (Altpeter, unpublished).
11. The removal of the selectable marker or reporter genes from transgenic elite events can be achieved by *Agrobacterium*-mediated co-transformation of unlinked T-DNA's followed by segregation analysis in sexual progenies (*12,13*).

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7

Switchgrass (*Panicum virgatum* L.)

Mariya N. Somleva

Summary

During the last decade, *Agrobacterium*-mediated transformation of more than a dozen monocotyledonous plants, including forage and turf grasses, has been achieved. So far, switchgrass is the only warm season grass that has been transformed with *A. tumefaciens*. We have developed a highly efficient system for transformation of different switchgrass explants utilizing the *A. tumefaciens* strain AGL1 carrying the binary vector pDM805, containing the phosphinotricin acetyltransferase (*bar*) and β-glucoronidase (GUS) (*uid*A or *gus*) genes. Transformed cultures were selected in the presence of 10 mg/L bialaphos and the resultant plantlets were treated with the herbicide Basta®. The T-DNA delivery frequency was affected by the genotype, explant used, and the presence or absence of acetosyringone during inoculation and cocultivation. The total time required from inoculation to the establishment of plants in soil was 3–4 mo. Stable integration, expression, and inheritance of both transgenes were confirmed by molecular and genetic analyses. Approximately 90% of the tested plants appeared to have only one or two copies of the T-DNA inserts. The transgenes were sexually transmitted through both male and female gametes to the progeny obtained from controlled crosses in the expected segregation ratio of 1:1 according to a χ^2 test at $p = 0.05$.

Key Words: *Agrobacterium tumefaciens*; Basta; bialaphos; *bar*; genetic transformation; GUS; *Panicum virgatum* L.; switchgrass; *uid*A.

1. Introduction

Currently, over 117 species of grasses are used for turf, forage, and erosion control, with new species being evaluated every year. Transgenic plants have been reported for only 6 genera and 11 species ***(1)***; the only warm season grass that has been transformed with *Agrobacterium* is switchgrass.

Switchgrass (*Panicum virgatum* L.) is a perennial C_4 grass that is native to the prairies and plains of North America ***(2)***. It has recently received interest for its potential as a bioenergy crop because of its high biomass production, low water

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and nutrient requirements, and eco-friendly attributes *(3,4)*. Important to the improvement of this species is the development of biotechnological approaches, including gene transfer that can be used to supplement conventional breeding programs.

It is a generally accepted notion that the availability of reproducible systems for plant regeneration is a prerequisite for efficient genetic transformation. Protocols for switchgrass regeneration via organogenesis and/or somatic embryogenesis from caryopses and young seedling explants *(5,6)*, in vitro developed inflorescences *(7)*, nodal segments *(8)*, suspension cultures *(9)*, and multiple shoot production from young seedlings *(10)* have been established. A highly efficient and reliable procedure for *Agrobacterium*-mediated transformation of switchgrass based on some of these regeneration systems has been reported *(11)*. A detailed transformation protocol is presented in this chapter. The hypervirulent *A. tumefaciens* strain AGL1 *(12)* carrying the cereal transformation vector pDM805 *(13)* was used for transformation of embryogenic calluses, somatic embryos, mature caryopses, and seedling segments. The plasmid contains the *bar* gene under the control of the maize ubiquitin 1 (*Ubi1*) promoter and the *uidA* or *gus* gene driven by the rice actin 1 (*Act1*) promoter. Various factors such as genotype, type of tissue used for inoculation, preculture of explants, wounding of tissues prior to infection, presence or absence of acetosyringone during inoculation and cocultivation, and different methods of selection have been found to influence switchgrass transformation. Generally, these factors are important for *Agrobacterium*-mediated transformation in most monocotyledonous plants *(14)*. Although the switchgrass genotypes used for transformation were all within the cultivar Alamo, there were differences for recovering transformed plants. Our results also revealed that the type of initial explants used for callus initiation affected transgenic plant production. Mature caryopsis-derived calluses showed the best regeneration potential among the embryogenic cultures utilized in these experiments, thus resulting in higher transformation efficiency. Therefore, the protocol for callus initiation from mature caryopses is included in this chapter. Among all the explants tested somatic embryos were the best targets for *Agrobacterium*-mediated gene transfer. Somatic embryos can proliferate highly embryogenic calluses, each of which produces numerous transformed plants during the selection process. Also, most of the recovered plantlets were transgenic, whereas approx 30% of the plantlets obtained from calluses were untransformed escapes. Stimulation of *Agrobacterium vir* system by acetosyringone increased the frequency of transgenic plants recovered, especially from somatic embryos, and improved the efficiency in most of the genotypes utilized. This protocol is successfully being used for transformation of different switchgrass genotypes, cv. Klanow (C. Tobias, USDA-ARS, Albany, CA; personal communication).

The procedure described here results in one of the highest frequencies for recovering transgenic plants reported for the *Agrobacterium* method in grass and cereal species. The average transformation efficiency determined in our system as the number of herbicide resistant plants recovered per explant inoculated was between 14 and 24% for calluses and somatic embryos, respectively. The success results from the virulence of the *Agrobacterium* strain (AGL1), the susceptibility of the target tissues as well as the promoters driving the transgene expression in pDM805. The application of Basta to young plantlets allowed early elimination of most of the untransformed escapes, thus additionally improving the efficiency of the whole procedure.

2. Materials

2.1. Plant Tissue Cultures

1. Mature caryopses (dry seeds) from the switchgrass cv. Alamo (USDA, Knox City Plant Materials Center, Accession 422006/Alamo).
2. Murashige and Skoog (MS) medium for callus initiation and maintenance: MS *(15)* salts and vitamins mixture (cat. no. 10632-022; Gibco) supplemented with 22.5 µ*M* 2,4-D, 5 µ*M* BA, 30 g/L maltose, and 8 g/L agar, pH 5.5.
3. MS medium for plant regeneration: basal MS medium supplemented with 1.4 µ*M* gibberellic acid (GA_3), 30 g/L maltose, and 8 g/L agar, pH 5.5.
4. Sterilization solution: 2.63% sodium hypochlorite (50% [v/v] commercial bleach) containing 0.1% (v/v) Triton X-100.
5. 60% (v/v) H_2SO_4.

2.2. Preparation of Agrobacterium Inocula

1. YEB medium (per L): 1 g yeast extract, 5 g beef extract, 5 g peptone, 0.5 g $MgSO_4 \cdot 7H_2O$, 5 g sucrose, pH 7.2. For *Agrobacterium* strain used in this protocol, supplement with 20 mg rifampicin and 5 mg tetracycline.
2. MG/L medium (per L): 5 g tryptone, 2.5 g yeast extract, 5 g mannitol, 1 g L-glutamic acid, 250 mg KH_2PO_4, 100 mg NaCl, 100 mg $MgSO_4 \cdot 7H_2O$, 1 µg biotin. For *Agrobacterium* strain used in this protocol, supplement with 20 mg rifampicin and 5 mg tetracycline.
3. 15% (v/v) aqueous glycerol solution, filter-sterilized.
4. 1% (w/v) stock solution of acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone). Dissolve in dimethyl sulfoxide (DMSO) and adjust the volume with MQ water. Store the filter-sterilized solution at 4°C, in a dark container. Add to the autoclaved medium.

2.3. Selection of Bialaphos-Resistant Callus Lines and Putative Transformants

1. Selection medium for transformed cultures: MS medium for callus initiation and maintenance supplemented with 150 mg/L timentin and 10 mg/L bialaphos.

2. Selection medium for plantlets obtained from selected cultures: MS medium for plant regeneration supplemented with 150 mg/L timentin and 10 mg/L bialaphos (*see* **Note 1**).
3. 0.1% (v/v) solution of the herbicide Basta® [monoammonium 2-amino 4 (hydroxy-methylphosphynil)butanoate].
4. Soil: a mixture consisting of 40% Canadian peat, 40% coarse vermiculite, 15% masonry sand and 15% screened topsoil, amended with 7.5 lbs Waukesha fine lime per cubic yard.

3. Methods

3.1. Initiation and Maintenance of Embryogenic Callus Cultures From Mature Caryopses

1. Dehusk whole caryopses with 60% H_2SO_4 for 30 min in a glass flask on a rotary shaker (*see* **Note 2**).
2. Discard H_2SO_4 and add the sterilization solution. (Work in a fume hood!) Incubate for 30 min with shaking.
3. Rinse with sterile water three times, for 5 min each.
4. Plate the sterilized caryopses onto callus induction medium and grow at 28°C, in the dark.
5. To maintain the cultures transfer calluses with formed somatic embryos onto a fresh induction medium every 4 wk and grow under the same conditions (*see* **Note 3**).

3.2. Preparation of A. tumefaciens *Inocula*

1. Streak *Agrobacterium* culture on YEB agar medium containing selective antibiotics and incubate at 28°C for 48 h in the dark.
2. Transfer a single colony of *A. tumefaciens* to 20 mL of MG/L medium with selective antibiotics and incubate on a rotary shaker (100 rpm) at 28°C for 40 h.
3. Aliquot 0.2 mL of the culture, mix with 0.2 mL of sterile 15% aqueous glycerol solution and store at –80°C.
4. For preparation of a full strength inoculum of *A. tumefaciens,* transfer the standard inoculum (0.4 mL) to 10 mL of MG/L medium supplemented with or without acetosyringone and incubate overnight at 28°C on a rotary shaker (100 rpm) in the dark (*see* **Note 4**).
5. Pellet bacterial cells by centrifugation for 5 min at 1000*g*.
6. Resuspend the pellet (OD_{600} = 0.5–0.6) in a liquid MS medium without plant growth regulators containing acetosyringone at the same concentration used for stimulation of the *Agrobacterium* virulent system in the overnight culture.

3.3. Explant Inoculation and Cocultivation

1. Aliquot *A. tumefaciens* full strength inoculum in a multiwell plate (1.5 mL/well).
2. Transfer approximately 25 immature somatic embryos or 10–15 callus pieces (2 × 2 mm each) into *A. tumefaciens* suspension (*see* **Note 5**).
3. Incubate for 3–10 min at 28°C, in the dark, with gentle shaking (*see* **Note 6**).

4. Transfer the explants with a wide-mouth pipet or with forceps onto MS medium for callus maintenance and culture at 28°C, in the dark for 3–5 d (*see* **Note 7**).
5. The effect of acetosyringone on transformation efficiency of mature caryopsis-derived embryogenic calluses and somatic embryos obtained from them is presented in **Table 1**.

3.4. Selection of Infected Cultures and Putative Transformants

1. After cocultivation, transfer cultures onto callus maintenance medium supplemented with 150 mg/L timentin and 10 mg/L bialaphos (*see* **Note 8**).
2. Transfer the cultures onto a fresh medium for selection of bialaphos-resistant callus lines every 2 wk for 4–6 wk (*see* **Note 9**). Selection of transformed callus lines and GUS activity in somatic embryos formed from them are shown on **Fig. 1**.

3.5. Plant Regeneration

1. Transfer the bialaphos resistant calluses to MS medium for plant regeneration supplemented with 150 mg/L timentin and 10 mg/L bialaphos. Incubate the plates at 28°C with a 16-h photoperiod, cool white fluorescent bulbs at 80 µmol/m^2/s (*see* **Note 10**), for about 4–6 wk with biweekly subcultres.
2. Transfer plantlets with 4–6 leaves to Magenta boxes (Sigma) containing 50 mL of regeneration medium with 150 mg/L timentin and 10 mg/L bialaphos. Grow plantlets under the same conditions as **step 1**.
3. After 2–3 wk, transfer the plantlets to 1-L polypropylene culture vessels containing the same medium (*see* **Note 1**).
4. After another 2–3 wk, transfer the plantlets to moist peat pellets, cover with plastic bags and grow for 2–3 more wk before transferring the plants to soil.

3.6. Greenhouse Care of Transgenic Plants

1. Grow plants in 16” pots containing the soil mixture described in **Subheading 2.3., step 4**.
2. Maintain the following conditions: 26–30°C/22–26°C day/night temperatures and a 16-h photoperiod using supplemental lighting from halide lamps (200 µmol/m^2/s).
3. Water with tap water as needed. Water with a nutrient solution containing 200 ppm N biweekly.
4. For control crosses, place pots with flowering transgenic and nontransgenic (control) Alamo plants next to each other on greenhouse benches and bag together their inflorescences. Harvest dry seeds.

3.7. Molecular Analysis

1. For Basta treatment under in vitro conditions, gently rub plantlets with 3–4 leaves with a sterile Q-tip soaked with 0.1% solution of Basta. Evaluate the effect after 3–5 d (*see* **Note 11**).
2. To test herbicide tolerance of adult plants, treat the upper part of at least one leaf of each tiller as described in step 1. Mark the treated leaf area and check the changes in the color of the tissues after 5–7 d.

Table 1
Effect of Acetosyringone (AS) on the Efficiency of *Agrobacterium*-Mediated Transformation of Somatic Embryos and Mature Caryopsis-Derived Embryogenic Calluses From a Switchgrass Genotype, cv. Alamo

Type of inoculated explants	AS concentration during inoculation (μ*M*)	AS concentration during cocultivation (μ*M*)											
		0				50				200			
		E No.	C No.	P No.	P/E %	E No.	C No.	P No.	P/E %	E No.	C No.	P No.	P/E %
Somatic embryos	0	54	32	0	0	20	6	0	0	24	5	8	33.3
	50	40	5	0	0	57	37	49	86.0	24	8	3	12.5
	200	50	8	0	0	24	6	0	0	74	41	72	97.3
Calluses	0	108	41	69	63.9	61	27	0	0	51	28	8	15.7
	50	108	29	11	10.2	131	48	10	7.6	59	31	6	10.2
	200	49	42	0	0	19	27	0	0	117	67	5	4.3

Notes: E, number of inoculated explants; C, number of bialaphos resistant calluses produced from them; P, number of Basta tolerant plantlets; P/E, transformation efficiency.

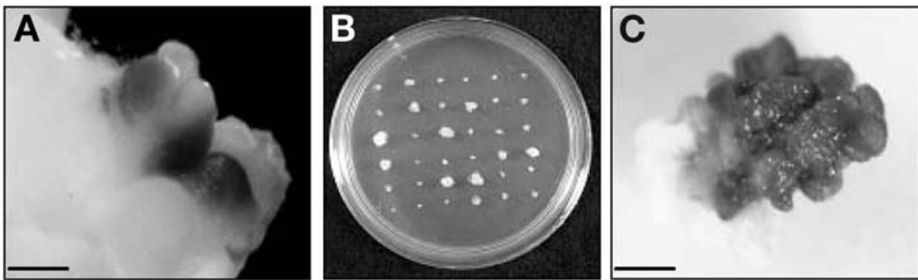

Fig. 1. *Agrobacterium*-mediated transformation of mature caryopsis-derived embryogenic cultures from switchgrass. **(A)** GUS activity (dark areas) in immature somatic embryos formed from a transformed callus one week after cocultivation. **(B)** Selection of calluses produced from inoculated somatic embryos in the presence of 10 mg/L bialaphos. **(C)** A callus with somatic embryos expressing the *uidA* gene after selection on a bialaphos-containing medium for 5 wk. Cultures were maintained for three months before transformation. Bar = 500 μm.

3. Stable integration, expression, and inheritance of both transgenes are confirmed by molecular and genetic analyses such as polymerase chain reaction (PCR), Southern, or GUS assay of primary transformants and their progeny obtained from controlled crosses.

4. Notes

1. The antibiotic can be omitted from the regeneration medium in the larger culture vessels, because *Agrobacterium* cells are completely eliminated at this stage of the selection process.
2. Switchgrass is highly self-incompatible; therefore seeds are produced by natural intercrossing of different genotypes. Because of the very small size of both the caryopsis and embryo (approx 800 seeds/g), it is too difficult to dissect seeds and isolate embryos as explants.
3. The best way to maintain the embryogenic capacity of long-term callus cultures is to transfer small groups of immature somatic embryos at globular to early scutellar stage onto a fresh medium at each subculture. In the presence of a high-auxin concentration they form a highly embryogenic callus that can be cultured for 10–12 mo.
4. Optimum acetosyringone concentrations depend on the target tissues.
5. Use the same procedure for transformation of embryogenic calluses initiated from other explants (i.e., immature inflorescences, seedling segments).
6. Inoculation in the bacterial suspension for up to 60 min does not cause significant changes in the frequency of transgenic plants produced. However, longer inoculation periods can result in *Agrobacterium* overgrowth during the subsequent cocultivation.
7. Optimum co-cultivation periods depend on target explants and are shorter for somatic embryos than for callus cultures.

8. Culture of inoculated explants on medium without or with low concentrations of bialaphos to favor multiplication of transformed cells results in more vigorously growing calluses but does not enhance the transformation efficiency. Rather, it allows for more untransformed escapes.
9. During subculture, divide each piece of callus derived from one somatic embryo or one piece of inoculated callus into several smaller pieces for better contact with the medium containing the selective agent.
10. One of the advantages of this switchgrass regeneration system is that both freshly initiated and long-term embryogenic callus cultures readily produce plantlets after transfer onto a regeneration medium. Another advantage is the ability of immature somatic embryos to develop to mature stages and regenerate on the same medium.
11. The sensitivity of leaf tissues from switchgrass plantlets to the herbicide Basta can be used to eliminate untransformed escapes at the beginning of the selection procedure. Basta treatment of developing leaves is also useful for detection of the *bar* gene expression in adult plants.

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8

Tall Fescue (*Festuca arundinacea* Schreb.)

Yaxin Ge and Zeng-Yu Wang

Summary

Tall fescue (*Festuca arundinacea* Schreb.) is the predominant cool-season perennial grass in the United States. It is widely used for both forage and turf purposes. This chapter describes a protocol that allows for the generation of large number of transgenic tall fescue plants by *Agrobacterium*-mediated transformation. Embryogenic calli induced from caryopsis are used as explants for inoculation with *A. tumefaciens*. The *Agrobacterium* strain used is EHA105. Hygromycin phosphotransferase gene (*hph*) is used as the selectable marker and hygromycin is used as the selection agent. Calli resistant to hygromycin are obtained after 4–6 wk of selection. Soil-grown tall fescue plants can be regenerated 4–5 mo after *Agrobacterium*-mediated transformation.

Key Words: *Agrobacterium*; *Festuca arundinacea*; forage and turf grass; tall fescue; transformation; transgenic plants.

1. Introduction

Tall fescue (*Festuca arundinacea* Schreb.) is the most important forage species worldwide of the *Festuca* genus. It forms the basis for beef cow–calf production in the east-central and southeast United States, supporting more than 8.5 million beef cows, and is also used for sheep and horse production ***(1)***. It is also widely used for general purpose turf and low-maintenance grass cover and plays an important role in environmental protection ***(2)***. The widespread use of tall fescue results from its adaptation to a wide range of soil conditions, tolerance of continuous grazing, high yields of forage and seed, persistence, long grazing season, compatibility with varied management practices, and low incidence of pest problems ***(1)***.

Tall fescue is a polyploid ($2n = 6x = 42$), wind-pollinated monocot species with a high degree of self-incompatibility. This makes breeding management difficult and selection schemes complex, resulting in slow breeding progress,

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especially for traits with low heritability *(3)*. There has been considerable interest in manipulating tall fescue by genetic transformation in the past decade with the aim of improving its agronomic traits *(4,5)*. In most of the cases, transgenic tall fescue plants were generated by direct gene transfer to protoplasts or by microprojectile bombardment *(4,6)*. Most of the transgenic plants obtained by protoplast or biolistic transformation had multiple copy insertions of the transgenes *(6)*. More recently, transgenic tall fescue has been obtained by *Agrobacterium*-mediated transformation *(6,7)*.

The protocol outlined in this chapter is based on our recent work *(6)* in tall fescue transformation. We use embryogenic calli as explant and hygromycin as selection agent. Transformation frequency is about 8.7% based on the number of transgenic plants recovered and the number of original intact calli used. The use of highly embryogenic calli is one of the key factors affecting transformation frequency.

2. Materials

2.1. Plant Material

Seeds of tall fescue cultivar Jes p *(8)* (*see* **Note 1**).

2.2. Agrobacterium tumefaciens Strain and Selectable Marker

The *Agrobacterium tumefaciens* strain EHA105 (*see* **Note 2**) is used in combination with the binary vector pCAMBIA1305.1, which carries a hygromycin phosphotransferase gene (*hph*) and a β-glucuronidase gene (*GUS*) (*GUS Plus* from *Staphylococcus* sp.), both under the control of CaMV 35S promoter (*www.cambia.org*). Hygromycin is used as selection agent.

2.3. Culture media for Agrobacterium tumefaciens

1. Luria Bertani (LB) medium: LB-Agar capsules (Qbiogene, Montreal, Canada).
2. AB medium *(9)*: The AB medium (per L) contains 3000 mg K_2HPO_4, 1000 mg NaH_2PO_4, 1000 mg NH_4Cl, 300 mg $MgSO_4 \cdot 7H_2O$, 150 mg KCl, 10 mg $CaCl_2 \cdot 2H_2O$, 2.5 mg $FeSO_4 \cdot 7H_2O$, and 5000 mg glucose. Autoclave.

2.4. Tissue Culture

1. Calcium hypochlorite: Prepare fresh 3% (w/v) calcium hypochlorite solution in a glass bottle; add a few drops of Tween-80.
2. 2,4-Dichlorophenoxy-acetic acid (2,4-D): Prepare 100 mg/L stock by dissolving the powdered chemical in a few drops of 1*M* KOH and add double distilled water (ddH_2O) to volume.
3. M5 medium: Murashige and Skoog (MS) medium (PhytoTechnology Laboratories, Shawnee Mission, KS) supplemented with 5 mg/L 2,4-D, 3% (w/v) sucrose and solidified with 0.8% (w/v) agar (Agar-Agar, Sigma). Adjust pH to 5.8 and autoclave.

4. AA medium (***10***): The modified AA medium (per L) contains 2950 mg KCl_2, 170 mg KH_2PO_4, 370 mg $MgSO_4 \cdot 7H_2O$, 440 mg $CaCl_2 \cdot 2H_2O$, 0.83 mg KI, 6.2 mg H_3BO_3, 16.9 mg $MnSO_4 \cdot H_2O$, 8.6 mg $ZnSO_4 \cdot 7H_2O$, 0.25 mg $NaMoO_4 \cdot 2H_2O$, 0.025 mg $CoCl \cdot 6H_2O$, 0.025 mg $CuSO_4 \cdot 5H_2O$, 27.8 mg $FeSO_4 \cdot 7H_2O$, 37.2 mg Na_2 ethylene diamine tetra acetic acid (EDTA), 100.0 mg inositol, 1.0 mg nicotinic acid, 1.0 mg pyridoxine HCl, 10.0 mg thiamine, 877 mg L-glutamine, 266 mg L-asparagine, 174 mg L-arginine, 7.5 mg glycine, 1.5 mg 2,4-D, 20 g sucrose, 25 g sorbitol, and 100 µ*M* acetosyringone. Adjust pH to 5.8 with KOH and filter-sterilize.
5. Acetosyringone (ACROS Organics, Morris Plains, NJ): Prepare fresh 100 m*M* acetosyringone (3,5-Dimethoxy-4-hydroxyactophenone) by dissolving acetosyringone in dimethyl sulfoxide (DMSO).
6. Hygromycin (EMD Bioscience, La Jolla, CA): Prepare 50 mg/mL stock in ddH_2O, filter sterilize and store at −20°C.
7. Cefotaxime (Agri-Bio, North Miami, FL): Prepare 250 mg/mL stock in ddH_2O, filter sterilize and store at −20°C.
8. M1 selection medium: MS medium (PhytoTechnology Laboratories) supplemented with 1.5 mg/L 2,4-D, 3% (w/v) sucrose and solidified with 0.8% (w/v) agar. Adjust pH to 5.8, autoclave, and then add 250 mg/L hygromycin and 250 mg/L cefotaxime.
9. Kinetin: 1 mg/mL stock (PhytoTechnology Laboratories).
10. MSK medium: MS medium (PhytoTechnology Laboratories) supplemented with 0.2 mg/L kinetin, 3% (w/v) sucrose and solidified with 0.8% (w/v) agar. Adjust pH to 5.8, autoclave, and add 250 mg/L cefotaxime.
11. MSO medium: Half-strength MS medium (PhytoTechnology Laboratories) solidified with 0.8% (w/v) agar. Adjust pH to 5.8 and autoclave.
12. Sterile distilled water.
13. Sterile filter paper (7-cm diameter).
14. Sterile 6.6-cm diameter (190 mL) plastic culture vessels (Greiner Bio-One, Longwood, FL).
15. Sterile 5.0-cm diameter (175 mL) plastic culture vessels (Greiner Bio-One, Longwood, FL).
16. Sterile plastic petri dishes, Parafilm.
17. Forceps, scalpel, and blades
18. Autoclaved glass flask (125 mL) and glass bottle (100 mL).
19. Drummond Pipet-Aid and sterile disposable pipets.
20. Magnetic stirrer and stir bars.
21. Shaker/incubator.
22. A rotary shaker.
23. Nalgene transparent polycarbonate desiccator.
24. A swing rotor centrifuge.
25. Spectrophotometer.
26. Metro Mix 350 soil (Sun Gro Horticulture, Terrell, TX).

3. Methods

3.1. Sterilization and Callus Induction

1. Immerse seeds in 3% calcium hypochlorite solution in a glass bottle. Put a magnetic stir bar in the bottle and place the bottle on a magnetic stirrer. Agitate for 2 h (*see* **Note 3**).
2. Rinse the seeds three times with sterile distilled water and leave the seeds overnight at 4°C.
3. Sterilize the seeds again for 30 min in 3% calcium hypochlorite solution the next day, rinse the seeds three times in sterile distilled water (*see* **Note 4**).
4. Place about 20 caryopses/seeds per 9-cm culture dish containing M5 medium. Keep dishes sealed with Parafilm and in the dark at 25°C.
5. Calli (Fig. 1A-C) formed within 5–7 wk are used for *Agrobacterium*-mediated transformation.

3.2. Agrobacterium *Preparation*

1. Streak *A. tumefaciens* from a glycerol stock onto LB agar plate with antibiotic selection appropriate for the vector used. Incubate at 28°C for 2 d.
2. Transfer a single colony from the plate into a flask containing 60 mL AB medium with antibiotic selection appropriate for the vector used.
3. Incubate the cultures on a shaker/incubator at 200 rpm at 28°C for about 2 d, until the culture has reached an OD_{600} of 0.8–1.2.

3.3. Inoculation of Explants and Cocultivation

1. Centrifuge the *Agrobacterium* culture at 2400*g* for 15 min.
2. Pour off supernatant, resuspend the pellet with AA medium, and adjust OD_{600} to 0.5. The *Agrobacterium* is now ready for transformation.
3. Transfer tall fescue calli into 6.6-cm culture vessels and break up the calli into small pieces.
4. Add *Agrobacterium* suspensions to the culture vessels and immerse the callus pieces.
5. Place the culture vessels in a polycarbonate desiccator and draw vacuum (62 cm Hg) for 5–10 min.
6. Release vacuum, incubate the callus pieces and *Agrobacteria* for 20 min with gentle shaking on a rotary shaker.
7. Remove the bacteria after the incubation, transfer the infected callus pieces onto M1 wetted filter papers placed in empty culture dishes in the dark at 25°C for cocultivation (*see* **Note 5**).

3.4. Selection and Plant Regeneration

1. After 2 d of cocultivation, transfer the filter papers supporting the infected callus pieces onto M1 selection medium.
2. After 2 wk, transfer the infected callus pieces to new M1 selection medium. Resistant calli normally become visible after 3–4 wk of selection (*see* **Fig. 1D**).

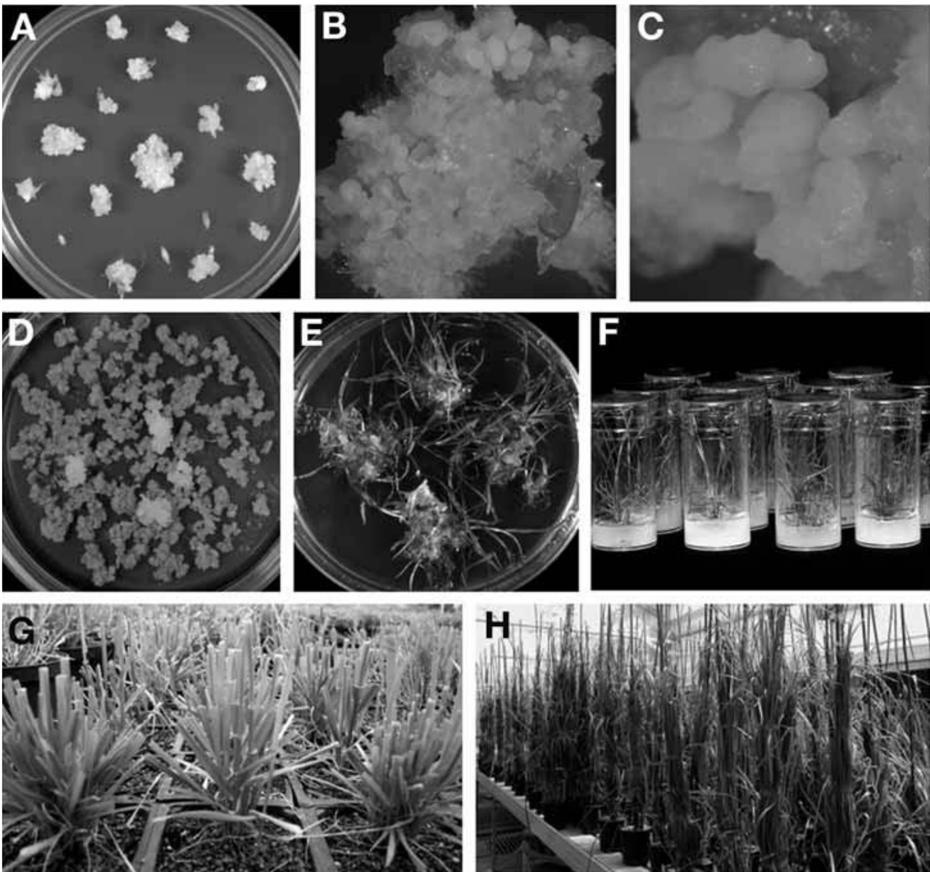

Fig. 1. Transgenic tall fescue (*Festuca arundinacea*) plants obtained after *Agrobacterium tumefaciens*-mediated transformation of embryogenic calli. (**A**) Embryogenic callus induced from caryopses. (**B**,**C**) Detailed view of the embryogenic calli. (**D**) Hygromycin resistant calli obtained 5 wk after *Agrobacterium*-mediated transformation and selection of infected callus pieces on M1 medium. (**E**) Shoot differentiation of hygromycin resistant calli 4 wk after transfer onto regeneration medium MSK. (**F**) Rooted transgenic plants 4 wk after transfer the differentiated shoots to rooting medium. (**G**) Greenhouse-grown transgenic plants 5 mo after *Agrobacterium* mediated transformation. (**H**) Fertile transgenic tall fescue after vernalization.

3. When the resistant calli are large enough (normally after 4–6 wk on M1), transfer the resistant calli onto regeneration medium MSK.
4. Keep the regenerating cultures at 25°C in fluorescent light (40 μE/m^2/s) at a 16-h photoperiod in the growth chamber for 4–6 wk (*see* **Fig. 1E**).
5. Transfer the regenerated shoots/plantlets (*see* **Fig. 1E**) to 5.0-cm culture vessels containing MSO medium (*see* **Fig. 1F**).

3.5. Greenhouse Care, Vernalization and Seed Harvest

1. Transfer well-rooted plantlets to 3 × 3 inch wells in an 18-well flat (6 × 3 wells) filled with Metro Mix 350 soil (*see* **Note 6**) and grow them under greenhouse conditions (390 μE/m^2/s, 16-h d/8-h night at 24°C/20°C) (*see* **Fig. 1G**). Plants can be grown on Ebb-Flo® benches and watered once a day with fertilized water containing 50 ppm N (Peters Professional 20:10:20 General Purpose is used as the water soluble fertilizer).
2. Transfer the established plants to 6-inch (1-gallon) pots filled with Metro Mix 350 soil and grow them under greenhouse conditions (390 μE/m^2/s, 16-h d/8-h night at 24°C/20°C).
3. For seed production, the plants need to be vernalized. Vernalization can be carried out by transferring the plants to the field in the autumn. However, growing transgenic plants in the field needs to be approved by regulatory agencies (e. g., USDA –APHIS).
4. Alternatively, transgenic plants can be vernalized in a cold room or growth chamber ***(11)***. The vernalization scheme involves gradual changes of temperature and daylength: (1) after transfer of greenhouse-grown plants to a cold room (31 μE/m^2/s), reduce temperature to 18°C and daylength to 12-h light and let the plants to adapt for 3 d; (2) reduce temperature to 12°C and daylength to 10-h and let the plants to adapt for 3 d; (3) reduce temperature to 6°C and daylength to 8 h and vernalize the plants for 12 wk; (4) increase temperature to 12°C and daylength to 10 h and grow the plants for 3 d; (5) increase temperature to 18°C and daylength to 12 h and grow the plants for 3 d; (6) transfer the vernalized plants back to the greenhouse (390 μE/m^2/s, 16-h day/8-h night at 24°C/20°C).
5. After vernalization, plants normally flower in about 2 mo (*see* **Fig. 1H**). Because tall fescue is an outcrossing species, crosses need to be made between independent plants (e. g., transgenic and nontransgenic plants) in order to obtain seeds. For cross pollination, emasculate recipient inflorescence and then bag them together with two panicles from the pollen donor plant; supply water to the donor panicles from a 50-mL conical tube fixed to a bamboo stake ***(12)***. Seeds can be harvested one month after cross pollination.

4. Notes

1. Transformation efficiency varies with the cultivar used. By using this protocol, we have been able to produce transgenic plants from another widely used cultivar, Kentucky-31. Endophyte-free seeds should be used.
2. We have been able to produce transgenic tall fescue with either EHA105 or LBA4404 strain.
3. If the seeds are dirty, the first sterilization time can be extended to 2.5 or even 3 h. Make sure all seeds have good contact with the solution.
4. The majority of seed bracts (lemma and palea) surrounding caryopsis become detached after the second sterilization. The surface sterilization procedure will not remove endophyte in the seeds. If the seeds contain endophyte, then the endophyte should be removed by treatment with high humidity and relatively high temperature. Older seeds contain less viable endophyte.

5. The amount of callus pieces on each filter paper was equivalent to approximately 20 original intact calli.
6. Before transfer to soil, rinse the roots with water or remove excessive medium with a damp paper towel.

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9

Turf Grasses

Chip Longo, Colin Lickwar, Qian Hu, Kimberly Nelson-Vasilchik, David Viola, Joel Hague, Joel M. Chandlee, Hong Luo, and Albert P. Kausch

Summary

A reliable and efficient genetic transformation protocol for various turfgrass species and elite cultivars has been achieved using *Agrobacterium tumefaciens*. We describe a general protocol for the establishment of embryogenic cell cultures, *Agrobacterium tumefaciens*-mediated transformation, selection, and regeneration of transgenic turfgrass plants. Embryogenic callus is initiated from mature seeds, maintained by visual selection, and infected with an *Agrobacterium tumefaciens* strain (LBA4404) that contains either an herbicide-resistant *bar* gene or an antibiotic-resistant *hyg* gene driven either by a rice ubiquitin or CaMV35S promoter. Stable transformation efficiencies up to 43.3% were achieved. Southern blot and genetic analysis was used to confirm transgene integration in the turfgrass genomes and normal transmission and stable expression of the transgene in the T_1 generation. We demonstrate herein that five elite cultivars of bentgrass can be genetically transformed using this single tissue culture media regime. Additionally, we report the successful *Agrobacterium*-mediated transformation of an elite tall fescue variety using minor variations in the same transformation protocol.

Key Words: *Agrobacterium tumefaciens*; creeping bentgrass; herbicide resistance; transformation; velvet bentgrass; colonial bentgrass; tall fescue.

1. Introduction

The improvement of turfgrass species through conventional breeding usually relies on the identification of a single improved trait within a cultivar, and is restricted to germplasm that is capable of sexual crosses to yield fertile offspring. An improved trait within a given cultivar, once identified, must be followed by extensive back-crossing, selection, and evaluation to produce a viable product for the commercial industry. Similarly, introgression of transgenes is also a time

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consuming process, hence, cultivar independent transformation of elite varieties is highly desirable for turfgrasses.

The development of general and efficient methods for genetic transformation of elite turfgrass cultivars is critical to the success of programs targeting trait modification for improved agronomic performance. Beneficial traits such as herbicide and fungus resistance to reduce chemical use, drought and stress tolerance that will reduce water usage, insect and pest resistance that will cut pesticide applications, phyto-remediation of soil contaminants, and horticultural qualities such as aluminum tolerance, stay-green appearance, pigmentation, and growth habit are among a long list of others that can be improved in turfgrass using genetic modification.

Agrobacterium-mediated transformation was first successfully applied to a wide range of dicotyledonous plant species ***(1)***. More recently, *Agrobacterium*-mediated transformation of various cereal plants ***(2–18)*** and grasses ***(19–23)*** has been achieved using modified binary vectors, various types and stages of plant tissue as well as modified transformation and culture parameters.

Initial reports of transgenic turfgrass utilized biolistics for gene transfer ***(24–26)***. *Agrobacterium*-mediated transformation is advantageous since it results in low-copy-number insertion events with fewer rearrangements and expression instabilities than by direct DNA delivery methods ***(9–14,23)***. The first reported attempt at *Agrobacterium*-mediated transformation of creeping bentgrass (*Agrostis stolonifera* L.) used green fluorescent protein (GFP) as a reporter gene and resulted in only a limited number of independent transgenic lines ***(19)***. Stable *Agrobacterium*-mediated transformation of turfgrass was first described by Barbara Zilinskas ***(20)*** and has since been substantiated by a number of laboratories ***(21– 23)***. The Zilinskas protocol relies on a callus induction medium (MMSG) that we have utilized for several turfgrass cultivars to derive embryogenic callus.

To efficiently recover transgenic colonies of cells after gene transfer, selectable marker genes are employed for positive selection. The *bar* gene, encoding phosphinothricin (PPT) acetyltransferase (PAT), has been shown to be an excellent selectable marker for grasses ***(20–26)***. The *hyg* gene has been effectively used as a selectable marker for rice ***(9,10)*** and grasses ***(20)***.

We describe a single standard method to efficiently produce large numbers of transgenic plants from five elite turfgrass cultivars using *Agrobacterium*-mediated transformation and both *bar* and *hyg* as selectable markers. Minor variations in this method have also been successfully applied to an elite tall fescue cultivar. This protocol results in high frequencies of single-copy foreign gene stable integration, expression, and inheritance. The transformation efficiency for bentgrass in our experiments ranged from 1.38 to 43.33%. This percentage represents the number of transgenic plants regenerated from the total number of selected putative transformed calli. From the total number of callus pieces treated with

Agrobacterium, 6.4% regenerate transformed plants (6.4 transgenic plants/100 infected calli). The average efficiency for tall fescue was comparatively lower than the bentgrass cultivars. Numbers of transformed calli were observed to be high (22.17 and 33.56%) from the total number of calli pieces infected (*see* **Table 1**) but regeneration of the calli was low (0.36 and 1.94%). These results could be attributed to the age, health, and cell-type used in the experiments. In bentgrass cultivars, high transformation rates were observed when calli were sub-cultured onto fresh media rigorously at 2- to 3- wk intervals and embryogenic calli were selected under a dissecting microscope. The results in the tall fescue experiments could be explained by cultures with nonembryogenic cell types that are transformable with *Agrobacterium* because of the ability of this cell-type to survive through selection, but nonregenerable. A high percentage of transformed calli were then observed, but most could not be regenerated.

2. Materials

2.1. Plant Materials

Commercial cultivars of creeping bentgrass (*Agrostis stolonifera* L., cv. Penn-A-4), colonial bentgrass (*Agrostis tenuis* cvs. Glory, PST, and 9F7), velvet bentgrass (*Agrostis canina* L., cv. Greenwich), and tall fescue (cv. Matador) were supplied by Turf-Seed, Inc. (Hubbard, OR) and used throughout this study. Seeds were stored at 4°C until used.

2.2. Bacterial Strains and Plasmids

Agrobacterium strains LBA4404 ***(27)*** were used for all of our experiments. This strain contains derivatives of the binary vector pSB11 ***(9)***. The T-DNA regions of these pSB11 derivatives are shown in **Fig. 1**. The Rice Ubi-*bar* and the CaMV35S-*bar* are vectors for using herbicide resistance (PPT) as the positive selectable marker. The pUbi-*gus*/Act1-*hyg* ***(23)*** construct was provided by Dr. Barbara Zilinskas (Rutgers University). This construct consists of the maize ubiquitin (*ubi*) promoter driving an intron-containing β-glucuronidase (*gus*) reporter gene, and the rice actin 1 promoter driving a hygromycin (*hyg*) resistance gene.

2.3. Tissue Culture Media Solutions

2.3.1. Stock Solutions (see **Note 1***)*

1. 3,6-Dichloro-*o*-anisic acid (dicamba): Stock concentration is made with 6.6 mg/mL double distilled water (ddH_2O) to use 1 mL of stock/L of media. Stock solution is stored at 4°C.
2. 6-benzylaminopurine (BAP): Stock concentration is made with 0.5 mg/mL ddH_2O to use 1 mL of stock/L of media. Stock solution is stored at 4°C.
3. Cefotaxime: Stock concentration is made with 125 mg/mL ddH_2O, filter sterilize and store at –20°C.

Table 1
Transformation Efficiency Data for Turf Species Tested

Plant variety	Experiment	Number of callus pieces inoculated	Resistant calli to R1 media	Number of plants regenerated	% Transgenic plants regenerated from R1	% Transformation from total calli inoculated
PennA4	1	4000	151	65	43.33*	3.78
PennA4	2	4000	645	86	13.33	16.13
PennA4	3	4000	145	2	1.38	3.63
PennA4	4	4000	71	0	0.00	1.78
PennA4	5	4000	672	33	4.91	16.80
PennA4	6	4000	8	8	100.00	0.20
PennA4	7	4000	247	40	16.19	6.18
PennA4	8	4000	110	28	25.45*	2.75
PennA4	9	4000	465	7	1.51	11.63
PennA4	10	4000	452	77	17.04	11.30
PennA4	11	4000	85	2	2.36	2.13
PennA4	12	4000	236	85	36.02*	5.90
PennA4	13	5571	248	36	14.52	4.45
PennA4	14	7560	388	122	31.44*	5.13
PennA4	15	5102	217	13	5.99	4.25
PST	1	4000	129	5	3.88	3.23
PST	2	4000	106	0	0.00	2.65
Greenwich	1	4000	901	3	0.33	22.53
Glory	1	1080	492	0	0.00	45.56
Glory	2	2775	837	9	1.08	30.16
Glory	3	3553	281	11	3.91	7.91
9F7	1	3606	333	2	0.61	9.23
Tall Fescue	1	4050	1359	26	1.94	33.56
Tall Fescue	2	7500	1663	6	0.36	22.17

* Indicates embryogenic callus selection using a dissecting microscope.

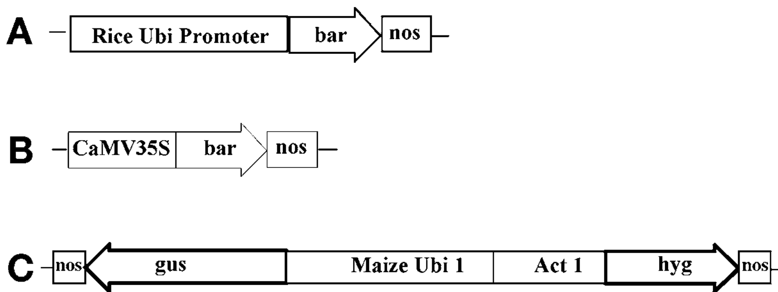

Fig. 1. Diagrams of the plasmid constructs used for turfgrass transformation. Plasmids with the Rice Ubi:bar **(A)**; CaMV35S:bar **(B)** are vectors utilizing *bar* as a selectable marker for PPT selection. Plasmid pUbi-gus/Act-hyg **(C)** contains a Maize Ubi promoter-driving reporter *gus* gene and a rice actin promoter-driving hygromycin resistance gene for selection.

4. Carbenicillin: Stock concentration is made with 125–250 mg/mL ddH_2O. Store at –20°C.
5. Phosphinothricin (PPT, Duchefa): Stock concentration is made with 10 mg/mL ddH_2O, filter sterilize, store at 4°C.
6. Hygromycin: Stock concentration is made with 200 mg/mL ddH_2O, store at 4°C.
7. Acetosyringone: Stock concentration is made with 100 mg/mL ddH_2O, filter sterilized, and stored at 4°C.

2.3.2. Induction of Embryogenic Callus From Seeds

1. Seed sterilization solution: Clorox® bleach (6% sodium hypochlorite) plus 0.2% (v/v) Tween-20™ (Polysorbate 20; PhytoTechnology Labs, Shawnee Mission, KS, USA). For creeping bentgrass, use 10% (v/v) bleach solution; for the other varieties of grasses used in this chapter, use 50% (v/v) bleach solution.
2. Bentgrass callus-induction medium (MMSG): 4.3 g/L Murashige and Skoog (MS) basal salts (Sigma; ***28***), 1 mL 1000X Gamborg vitamins (Phytotechnology), 30 g/L sucrose, 500 mg/L casein hydrolysate, 6.6 mg/L 3,6-dichloro-*o*-anisic acid (dicamba), 0.5 mg/L BAP, and 2 g/L Phytagel. Adjust the pH of the medium to 5.7 using KOH or HCl before autoclaving at 123°C for 20 min.
3. Tall fescue callus-induction medium (MMS5D): 4.3 g/L MS basal salts, 1 mL 1000X Gamborg vitamins, 30 g/L sucrose, 500 mg/L casein hydrolysate, 5 mg/L 2,4-dichlorophenoxyacetic acid, 0.5 mg/L BAP, and 2 g/L Phytagel. Adjust the pH of the medium to 5.7 using KOH or HCl before autoclaving at 123°C for 20 min.

2.3.3. Agrobacterium *Medium*

1. YEP medium: 10 g/L bacto-peptone, 10 g/L yeast extract, 5 g/L sodium chloride, and 15 g/L agar. Autoclave at 123°C for 20 min. After cooling, add the appropriate antibiotics for vector selection depending on which strain of *Agrobacterium* used. Omit agar for liquid media.

2.3.4. Plant Selection and Regeneration Media

1. Selection media
   a. For bentgrasses: MMSG plus 125 mg/L cefotaxime and/or 125–250 mg/L carbenicillin and 10 mg/L phosphinothricin PPT (for *bar* selection), or 200 mg/L hygromycin (for *hyg* selection).
   b. For tall fescue: MMS5D plus 125 mg/L cefotaxime and/or 125–250 mg/L carbenicillin and 10 mg/L phosphinothricin PPT (for *bar* selection), or 200 mg/L hygromycin (for *hyg* selection).
2. Regeneration medium (R1) for shoot induction: MS basal medium, 30 g/L sucrose, 100 mg/L myo-inositol, 1 mL 1000X Gamborg vitamins, 1 mg/L BAP, pH 5.7, with KOH or HCL, and 2 g/L Phytagel. Sterilize the media at 123°C for 20 mins. After cooling the media to 58°C in a water bath, add 125 mg/L cefotaxime and/or 125–250 mg/L carbenicillin, and either 10 mg/L PPT, or 200 mg/L hygromycin.
3. Regeneration medium (R2) for root induction: MS basal medium, 30 g/L sucrose, 100 mg/L myo-inositol, 1 mL 1000X Gamborg vitamins, pH 5.7, and 2 g/L Phytagel. Sterilize the media at 123°C for 20 mins. After cooling the media to 58°C in a waterbath, add cefotaxime (125 mg/L) and/or carbenicillin (125–250 mg/L), and either 10 mg/L PPT, or 200 mg/L hygromycin.

3. Methods

3.1. Induction and Proliferation of Embryogenic Callus From Seeds

1. Dehusk mature seeds between two sheets of sandpaper and collect the cleaned seeds in a sterile, 50-mL Falcon tube (*see* **Note 2**).
2. When 10–15 ml of cleaned seed is obtained, add approx 50 mL 70% ethanol to the Falcon tube/tubes and vigorously shake (175–200 rpm) on an orbital shaker for 2 mins. Rinse seeds three times with sterile water. Retain seeds in sterile water after rinsing.
3. Fill the Falcon tube/tubes with seed sterilization solution. Shake the tubes vigorously (175–200 rpm) for 30 mins. Repeat this step two times. Once the Clorox® and rinsing is complete, the seeds are ready to be plated onto induction media.
4. Place the sterilized seeds onto callus-induction medium. For all cultivars of turf other than creeping bentgrass, place seeds (approx 40–50) on the medium carefully seed-by-seed with forceps, using sterile techniques under a laminar flow hood (*see* **Note 3**).
5. For small size creeping bentgrass seeds, retain the seeds in sterile water. Using a pipet, transfer a small number of seeds (approx 80–100) and 1 mL of the sterile water to the induction medium. Evenly spreading of the seeds on the surface of the plate by swirling plate. Spreading seeds facilitates sub-culturing after germination. Remove any extra water after the seeds are evenly spread.
6. The culture plates containing the seed explants are kept in the darkness at room temperature for 2–3 wk.

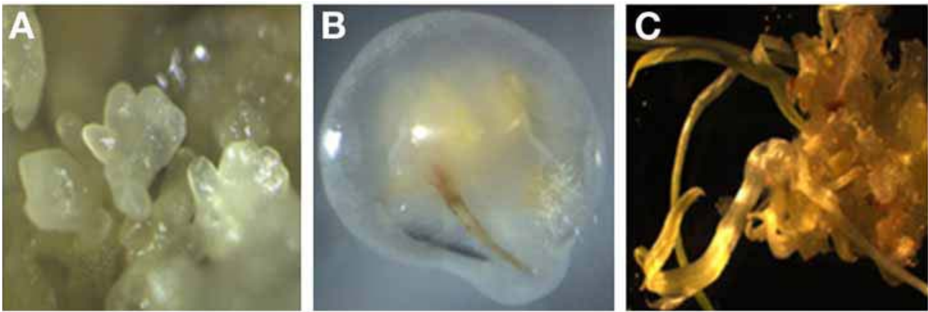

Fig. 2. Generation and selection of callus types associated with creeping bentgrass tissue culture. **(A)** Embryogenic calli visualized under a dissecting microscope at ×40. **(B)** 'Mucilaginous' callus type at ×20. **(C)** Differentiating 'leafy' callus type growing out of callus at ×20.

7. Once the seeds have germinated, sterile forceps are used to transfer germinated seedlings (approx 10–20) onto fresh callus induction media, allowing more space for growth. Cultures are then returned to the darkness for another 2–3 wk of growth.
8. Embryogenic calli (reminiscent of Type II callus of maize, *see* **Fig. 2A**) are visually selected and sub-cultured onto fresh callus-induction medium. Other callus morphologies, such as mucilaginous calli, differentiated callus types (similar to Type I callus of maize), and organized tissues such as roots and shoots (*see* **Fig. 2**) are discriminated against and discarded (*see* **Notes 4 and 5**).
9. All embryogenic cultures are returned to the darkness for growth and cell line expansion. Sub-culturing callus is repeated every 2–3 wk by 'fracturing' callus into 0.5-cm pieces. Additional medium space is allowed for continued growth of sub-cultured colonies. Sub-culturing is repeated until enough colonies (approx 1500 calli) are generated for a transformation experiment.

3.2. Callus Preparation for Agrobacterium *Infection*

1. Start preparing callus culture for infection 1–2 wk before transformation experiment. (*see* **Note 6**)
2. Sub-culture callus pieces (2 mm in size) to fresh callus induction media. The pieces are placed close together (50–60 pieces/plate) in straight rows to facilitate *Agrobacterium* application and placed in the darkness until infection.

3.3. Agrobacterium *Preparation*

1. *Agrobacterium* is taken from a –80°C glycerol stock and streaked onto YEP media plates with the appropriate antibiotics for correct transformed colony selection. Incubate the plates at 29°C for 2 d.
2. Under sterile conditions, a single colony is taken from the streaked YEP plate and put into 2 mL of YEP liquid media containing the appropriate antibiotics for the

Agrobacterium strain used. Shaker incubate the liquid culture (175–240 rpm) overnight at 29°C.

3. Depending on the total amount of callus used for a given transformation experiment, the appropriate amount (0.5–1.0 L) of YEP liquid with appropriate antibiotics, is inoculated with the 2 mL of YEP from **step 2** and then grown for 24–36 h at 29°C with shaking in darkness.
4. Cell density is determined by optical density OD_{600} with a reading of 1.1 to 1.4 indicating late log-phase growth which optimal for transformation.
5. Once the proper OD is reached, aliquots of the culture are dispensed to sterile 50-mL Falcon tubes and centrifuged at 3220*g* for 10 min.
6. After centrifugation, the YEP supernatant is discarded. The appropriate sterilized liquid callus induction media containing 100 mg/L acetosyringone is then added to the Falcon tube and the pellet is resuspended with gentle agitation by hand. The *Agrobacterium* suspension is adjusted to an OD_{600} of 1.1 to 1.4.

3.4. Agrobacterium *Transformation Protocol*

1. Day 1: The prepared calli are individually pre-treated (by 'spotting' with a micropipettor (*see* **Fig. 3B**) with 10–15 µL of sterilized liquid callus induction media containing 100 mg/L acetosyringone and returned to darkness overnight.
2. Day 2: The cultures are 'spotted' with 10–15 µL of the prepared *Agrobacterium* suspension and placed back in the dark for co-cultivation for 3 d.
3. Day 5: The infected callus pieces are transferred onto the appropriate selection media. Place calli with even spacing at 25 pieces/plate.
4. Once complete, the number of plates is tallied to determine the number of callus pieces used in the transfection experiment (25 pieces/plate × number of plates). Efficiency of transformation data is calculated from this number. The infected calli are then sub-cultured onto fresh selection media at 2–3 wk intervals for 6–10 wk (*see* **Fig. 3.** and **Notes 7** and **8**).

3.5. Selection of Transformed Colonies

1. Resistant colonies are visually apparent between 4 and 10 wk. The resistant colonies are removed and sub-cultured at 2–3 wk intervals using care to select only for transformed colonies (*see* **Note 9**).
2. In the first sub-culture passage, most of the resistant callus colony is moved to fresh selection media. For *bar* gene selection, PPT at 10 mg/L has a slower kill rate than hyromycin selection so calli are allowed an additional 2–4 wk on selection media to distinguish between transformed colonies and nontransformed colonies. Obvious dead material is discarded.
3. Each resistant colony is kept isolated far enough from others to ensure that mixing of independent transformation events does not occur. At each sub-culturing, transformed events are given ample space to allow growth without contaminating other independent events. Individual transformed colonies exhibit different growth rates and are therefore independently assessed for transition to regeneration based

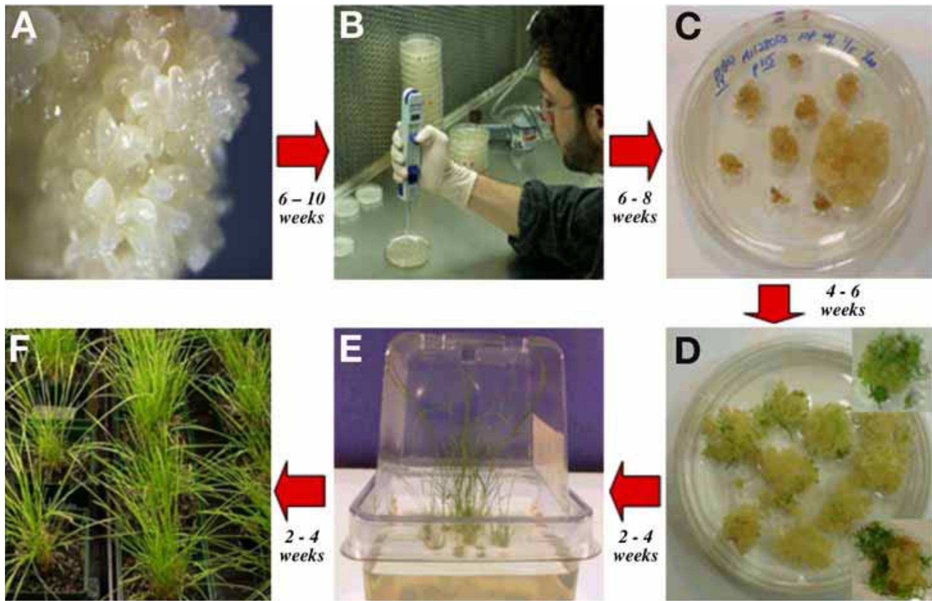

Fig. 3. Standard operating procedures for *Agrobacterium*-mediated transformation of turfgrass. **(A)** Induction and selection of embryogenic creeping bentgrass calli. **(B)** DNA delivery using *Agrobacterium tumefaciens* and the "spotting" method. **(C)** Selection of transformed calli using *bar* or *hyg* resistance. The colonies that do not carry the transgene die while putative transformants thrive and continue to grow under selection pressure. **(D)** Regeneration1 for transformed calli under continued selection pressure. **(E)** Regeneration 2 for transformed calli under continued selection pressure. **(F)** Regenerated transformed plants are placed into soil for further growth and development.

on size and a visual healthy appearance. Additional time may be required for the smaller and more slowly growing colonies prior to regeneration.

3.6. Plant Regeneration From Embryogenic Callus

1. Once the resistant colonies have proliferated to a diameter of approximately 1–2 cm, half of the colony is transferred to regeneration media (R1), while the remaining colony is transferred to fresh selection media and maintained as backup cultures.
2. The colonies are given a number on both R1 media and selection media for identification and tracking. This process is repeated for all transformed colonies transferred to R1 media. The R1 plates are incubated in darkness for 5 d at 28°C.
3. The callus pieces are then transferred into light for embryo germination and shoot development. A growth chamber is used with fluorescent lighting (250 $\mu E/m^2/s$) and a temperature of 28°C.
4. Shoots are observed as early as 2 wk after sub-culturing to R1 medium. Small shoots or resistant calli not yet showing regeneration can be transferred to fresh R1 media for additional development.

5. Shoots that are large enough (1–2 cm) are transferred to R2 media where they remain until roots developed (2–4 wk). A deep dish Petri plate (100 × 15 mm) is used to allow growth of the newly regenerating transgenic plants. As the plants grow they need additional space. Plantcons® or similar type containers are used to provide additional growth space for the shoots. If the transgenic plantlets do not develop roots within a 2-wk period, calli are transferred to fresh R2 media (*see* **Note 10**).

3.7. Plant Hardening Off and Greenhouse Maintenance

1. When regenerated plants have developed a robust root system, plants are then gently taken out of tissue culture with gloved hands, rinsed, and placed into soil (Pro-Mix®) in a 4-in pot.
2. Potted plants are labeled with experiment numbers and identification tracking numbers. Plants are placed in a light chamber at room temperature with fluorescent lighting (250 μE/m^2/s) with 16 h of light and 8 h of darkness with 85% relative humidity, and 600 ppm CO_2. Plants are covered with a PlantCon® cover for 5 d to prevent desiccation of nubile plants. Plants are then transferred to greenhouse conditions (75–80°C with natural light) where they are grown to maturity.
3. To obtain T_1 seeds, plants are stimulated to flower in the greenhouse as follows. Transgenic plants are grown in polyhouses at ambient temperatures through a summer growing season and maintained with a regular watering regime, treated every 4 wk with liquid soluble 20:20:20 fertilizer and cut to a height of 2–3 cm.
4. During the late autumn, the plants are allowed to proceed into dormancy as the temperature drops to freezing (0°C). After the winter solstice (around December 21) the plants are transferred to a greenhouse (28°C) with 16-h supplemental lighting (1000 μE/m^2/s).
5. Flowering occurs simultaneously in about 4 wk and transgenic plants are pollinated with wild-type donors. T_1 seed is recovered in 8 wk.

3.8. Molecular Analysis

To determine foreign gene insertions in T_0 regenerated plants and T_1 seed derived plants, the genomic DNA from leaf tissue of each independent transformation event is isolated as previously described *(**26**)* and analyzed by polymerase chain reaction (PCR) and Southern blot hybridization as described by Sambrook et al. *(**29**)*. We see very few (<3/1500) nontransgenic plants as resistant escapes when plantlets are regenerated in the presence of the selective agent. Transgenic efficiencies are shown in **Table 1**.

4. Notes

1. Some of the chemical components that are added to tissue culture media are heat sensitive. In order to avoid degradation of these components, stock solutions are added to tissue culture media under sterile conditions, when media is cooled in a water bath to 58–59°C. Stock solutions are filter sterilized and stored appropri-

ately prior to use. Stock solutions are made according to concentrations required per liter volume media.

2. This is not necessary for creeping bentgrass cv. PennA4 because of their size.
3. A high density (>100) of seeds per plate will result in inconsistent callus induction and difficult separation.
4. It is important to visually distinguish callus types and select for the healthiest embryogenic calli. A dissecting microscope may be helpful to distinguish callus morphologies. *See* **Fig. 2** for descriptions of embryogenic calli and other tissue types.
5. Creeping bentgrass embryogenic calli must be selected using a dissecting microscope under sterile conditions. Creeping bentgrass embryogenic calli are easily distinguished under these conditions, which allows for the production of nearly 'pure' line of cells. Although small at this age (<2 mm diameter), these pieces are easily recovered and proliferate rapidly after sub-culturing. This process is repeated to further select the most homogeneous embryogenic cell lines for transformation.
6. This helps to ensure that there is no contamination of the cultures and allows growth recovery of cultures from sub-culturing. Experiments can vary in size. A typical experiment will use between 5000–10,000 pieces of calli.
7. Transformation efficiency appears to be strongly correlated to callus health, time in culture, and embryogenic callus selection. Unhealthy callus can result in regenerated plants with compromised regeneration capacity and reproductive fertility compared to wild-type plants.
8. For all turf species tested, regular callus sub-culture passage every 2 wk is essential to culture health and transformation efficiency. After 4 wk on the same medium, callus is visually stressed and should probably not be used for an experiment.
9. Identification and selection of embryogenic calli under a dissecting microscope results in improved transformation and regeneration frequencies. This is indicated in the data shown in **Table 1**.
10. Maintenance of selection throughout plant regeneration is imperative for prevention of recovery of nontransformed plants.

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III

Woody Species

10

American Elm (*Ulmus americana*)

Andrew E. Newhouse, Franziska Schrodt, Charles A. Maynard, and William A. Powell

Summary

American elm (*Ulmus americana*) is a valuable and sentimental tree species that was decimated by Dutch elm disease in the mid-20th century. Therefore, any methods for modifying American elm or enhancing disease resistance are significant. This protocol describes transformation and tissue culture techniques used on American elm. Leaf pieces containing the midvein and petiole are used for explants. *Agrobacterium tumefaciens* strain EHA105 is used for transformation, with the binary vector pSE39, containing CaMV35S/*npt*II as a selectable marker, ACS2/ESF39A as a putative resistance enhancing gene, and CaMV35S/GUS as a reporter.

Key Words: American elm; *Ulmus Americana*; *Agrobacterium*; Dutch elm disease; transformation for disease resistance; antimicrobial peptide; tree improvement.

1. Introduction

American elm trees were an economically, socially, and ecologically important part of the eastern United States until Dutch elm disease killed most of the mature trees in the mid-20th century. They were commonly used in urban landscaping, and were valued for their drought and pollution tolerance, thick shade, and graceful vase shape as they grew over city streets. Treating trees with fungicides to prevent Dutch elm disease (DED) is expensive and laborious, and American elm's tetraploidy makes breeding with DED-resistant elm species difficult or impossible *(1)*. Some DED-resistant triploid hybrids (possibly *Ulmus parvifolia* × *U. americana*) have been observed, but *U. parvifolia* (Chinese elm) doesn't have the pleasing shape or cold-tolerance of a full-American elm, and progeny viability of these trees is low *(2,3)*. Screening programs to select naturally DED-resistant American elms were started by the United States Department of Agriculture (USDA) in 1937, and though they have produced

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several clones that can tolerate Dutch elm disease, progress has been slow and none of these are truly resistant *(3,4)*. Transformation of American elm trees could therefore be a very valuable tool to produce disease-resistant trees in a comparatively short timeframe while maintaining American elm characteristics not found in other elm species. Transgenes encoding antimicrobial peptides *(5–8)* under the control of a vascular promoter *(9)* could be potentially useful in protecting American elm trees against DED and other diseases such as elm yellows.

The first step in genetically engineering American elm trees was to develop a tissue culture procedure that could regenerate whole plants from transformed cells. Significant work had been completed on the tissue culture of various elm species *(10–23)*. The protocol detailed in this chapter is based on the first published method that showed regeneration of shoots from leaf slices of American elm *(24)*. This method could be used to regenerate shoots but it needed further optimization to develop a stage I shoot multiplication medium and a stage II shoot elongation medium. A matrix of varying concentrations of benzyladenine (BA) and indole butyric acid (IBA) was tested, resulting in the concentrations described in this protocol (Powell, unpublished data). Initially, prolific callus growth was a problem when regenerating shoots. Addition of silver nitrate significantly reduced callus growth and promoted shoot formation. A stage III root induction medium was developed using an auxin dip followed by growth in a charcoal medium. This was a simplified modification of a method developed for American chestnut *(25)*. Finally, the whole plants were acclimated by planting the rooted shoots in potting mix and slowly decreasing the humidity. This procedure enabled transformation of American elm.

Agrobacterium-mediated transformation has been used to transform English elm (*Ulmus procera*) *(20,26)*, the hybrid elm Pioneer (*Ulmus glabra* × *carpinifolia*) *(27)*, as well as American elm (Newhouse, Schrodt, Maynard, and Powell, unpublished*)*. This protocol has produced stable transformants, confirmed by Southern analysis, at a frequency of about 3 events/1000 American elm leaf pieces co-cultivated with *Agrobacterium* (Southern analysis data not shown*)*.

The main features of this protocol include growing *Agrobacterium* to late-log phase in a 0.5% sucrose minimal medium *(28)*, then diluting to a concentration of approximately 1×10^8 cells/mL. *Agrobacterium* is then transferred to an induction medium containing acetosyringone for *vir*-gene induction, and then to liquid co-cultivation medium also containing acetosyringone. The *Agrobacterium*-soaked leaf pieces are briefly sonicated and then cultured on a semi-solid elm tissue culture medium without selection. Finally, leaf pieces are rinsed with sterile, distilled water containing cefotaxime and carbenicillin to remove excess *Agrobacterium*, and are then grown on semi-solid tissue culture media with selection.

Co-transformation with multiple *Agrobacterium* strains is currently being tested on American elm as a method to pyramid resistance-enhancing transgenes. Co-transformation has been shown to incorporate the T-DNA from two cultures of *Agrobacterium* in about one third of the transformants of poplar (*29*). In this method, two (or more) strains of *Agrobacterium* are grown separately until the co-cultivation step, at which equal concentrations of each strain are mixed together and used to transform the plant. This variation on the basic protocol is potentially useful for introducing multiple gene constructs into a plant or for introducing "temporary" genes (i.e., markers or reporters) that could possibly be bred out later.

2. Materials

2.1. Media and Solutions

1. Bleach solution for leaf sterilization: 10% (v/v) Clorox® bleach in distilled water, 0.1% Tween-20 (i.e., about 10 drops/L). Make fresh, do not autoclave.
2. Luria–Bertani (LB) agar and broth with *Agrobacterium* selection: Add LB broth/agar (Fisher, DF0446-17-3, DF0445-17-4) to water as per manufacturer's instructions, autoclave, cool to 55°C, add 100 µg/mL kanamycin and 25 µg/mL rifampicin. Store up to 30 d in the dark at 4°C.
3. AB buffer (20X): 0.344 *M* K_2HPO_4, 0.145 *M* NaH_2PO_4. Autoclave and store indefinitely at 4°C.
4. AB salts (20X): 0.374 *M* NH_4Cl, 0.243 *M* $MgSO_4{\cdot}7H_2O$, 0.04 *M* KCl, 1.8 m*M* $CaCl_2$, 0.18 m*M* $FeSO_4{\cdot}7H_2O$, pH to 7 with KOH before autoclaving. Store up to 6 mo at 4°C (*see* **Note 1**).
5. AB Sucrose minimal media: 0.05 g sucrose in 90 mL of double distilled water (ddH_2O) (final concentration is 0.5% [w/v] Sucrose), autoclave, cool to 55°C or below, add 1X AB Salts (i.e., include 5 mL of 20X AB salts in 100 mL media), 1X AB Buffer, 100 µg/mL kanamycin, and 25 µg/mL rifampicin. Make fresh and keep 5 mL of each batch out to use as a spectrophotometer blank.
6. Induction media: 1X AB salts (i.e., 25 mL in 500 mL media), 2 m*M* NaH_2PO_4, 50 m*M* 2-(*N*-morpholino)ethanesulfonic acid (MES buffer, pH 5.6), (Phytotechnology Laboratories, cat. no. M825), 0.5% (w/v) glucose, and 100 µ*M* acetosyringone. Adjust pH to 5.6 with NaOH, and autoclave.
7. Co-cultivation liquid medium: 2.30 g/L McCown's woody plant basal salt mixture (WPM salts, Sigma, M6774), 5.9 m*M* $MgCl_2$, 2.6 m*M* MES Buffer, 6% (w/v) sucrose. Adjust pH to 5.2 with 1 *M* KOH. Add 200 µL/L of Vac-In-Stuff (Lehle Seeds, cat. no. VIS-01, Round Rock, TX). Autoclave and cool to below 55°C. Medium can be stored up to 1 mo at 4°C at this point. Use sterile stocks and add the following ingredients aseptically: 1.0 µ*M* *N*-(2-chloro-4-pryidyl)-*N*-phenylurea (4-CPPU, Phytotechnology Laboratories, no. C279), 200 µ*M* acetosyringone, 1 m*M* L-proline, 3.3 m*M* L -cysteine.
8. Elm shoot initiation media (ESI): 35.2 g/L of DKW basal salt mixture with 30 g/L sucrose (PhytoTechnology Laboratories, cat. no. D189), 1X Nitsch & Nitsch vitamin

powder (PhytoTechnology Laboratories, no. N608) (0.11 g/L), 0.55 m*M* L-tyrosine, 2.6 m*M* MES Buffer, 6.25 μ*M* Polyvinyl-Pyrrolidone (PVP-40, Sigma no. PVP-40), additional 20 g sucrose (final sucrose conc. 5% w/v), 29.6 μ*M* thiamine-HCl, 0.555 m*M* myo-inositol, 26.6 μ*M* glycine, 22 μ*M* BA, 0.1 μ*M* indole-3 butyric acid (IBA). Adjust pH slowly to 5.6±0.05 with 1M KOH. Add 0.8% (w/v) tissue culture-grade agar (Phytotechnology Laboratories, cat. no. A111), microwave until transparent. Autoclave, cool to 55°C, add post-autoclave ingredients from sterile stocks if necessary, and pour 20–25 mL into 110 × 15-mm Petri plates and let cool in sterile hood with lids ajar to reduce condensation. Five hundred millimeters of media is sufficient for 20–25 plates. Store at 4°C in the dark for up to 30 d.

9. ESI co-cultivation solid media: Same as ESI, but after autoclaving, add 1 m*M* L-proline, 100 μ*M* acetosyringone, and 3.3 m*M* L-cysteine. Mix well before pouring plates.
10. ESI selection media: Same as ESI, but after autoclaving, add 94 μ*M* $AgNO_3$, 250 mg/L cefotaxime, 200 mg/L carbenicillin, and the appropriate selection agent (100 μg/mL kanamycin for this construct).
11. Elm elongation media (EEL): 35.2 g/L of DKW basal salt mixture with 30 g/L sucrose, 1X Nitsch & Nitsch vitamin powder (0.11 g/L), 2.6 m*M* MES Buffer, 6.25 μ*M* PVP-40, 29.6 μ*M* thiamine-HCl, 0.555 m*M* myo-inositol, 26.6 μ*M* glycine, 5.5 μ*M* BA, 0.22 μ*M* IBA. Adjust pH slowly to 5.6±0.1 with 1*M* KOH. Add 0.8% (w/v) tissue culture-grade agar, microwave until transparent. If no post-autoclaving ingredients are needed, pour 45–50 mL into each short Magenta™ cube, cap, and autoclave. Leave caps ajar in sterile hood until medium solidifies to reduce condensation. Five hundred milliliters of media is sufficient for 10–11 cubes. If antibiotics are needed, autoclave media along with empty cubes, cool to 55°C, add cefotaxime, carbenicillin, and/or selection agent, then pour into sterile cubes. Keep up to 30 d in the dark at 4°C.
12. Elm rooting media (ERM): 35.2 g/L of DKW basal salt mixture with 30 g/L sucrose, 1X (0.11 g/L) Nitsch & Nitsch vitamin powder, 2.6 m*M* MES Buffer, 6.25 μ*M* PVP-40, 29.6 μ*M* thiamine-HCl, 0.555 m*M* myo-inositol. Adjust pH slowly to 5.6±0.1 with 1*M* KOH. Add 0.2% (w/v) activated charcoal powder and 0.8% (w/v) tissue culture-grade agar, microwave until agar dissolves (solution will not be transparent with charcoal, but consistency becomes more uniform as solution approaches boiling), pour about 100 mL into each tall Magenta cube, cap, and autoclave. One liter makes about 10 tall cubes. Keep up to 30 d at 4°C.
13. X-gluc: 5-Bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexylamine salt (Rose Scientific Ltd., cat. no. ES-1007-001)
14. Soil mixture: Fafard no. 2 Grower Mix (Griffin Greenhouse and Nursery Supply, cat. no. 65–1424)

2.2. Explant Source

Fresh, healthy American elm leaves. Seeds can be obtained from F.W. Schumacher Co., Inc. Horticulturalists (Sandwich, MA) (*see* **Note 2**).

2.3. Agrobacterium *Strain and Vector*

The binary vector construct tested with this protocol, called pSE39, includes three genes with separate promoters. First, the β-glucoionidose (GUS) gene (closest to right border of the T-DNA), driven by a CaMV35S promoter, is used as a reporter to confirm transformation. Our gene of interest for fungal resistance is called ESF39A, which codes for a synthetic antimicrobial peptide (*5,7,8*). This gene is controlled by ACS2, a vascular-specific promoter isolated from American chestnut (*Castanea dentata*) (*9*). Finally, in the opposite orientation to the previous genes, a separate CaMV35S promoter drives the selectable marker *npt*II.

2.4. Other Equipment for Transformation

1. Vortexer.
2. 27°C incubator.
3. Variable-speed rotary shaker that can be used inside a 27°C incubator.
4. Sonicator: Branson 2510 Ultrasonic Cleaner (Branson, Danbury, CT).
5. Nephelo flasks, 250 mL, 14 × 300 mm (Kimble/Kontes Glass Co., Vineland, NJ).

2.5. DNA Extraction Solutions and Materials

1. Liquid nitrogen.
2. Polyvinyl-pyrrolidone (PVP-40) (Sigma, cat. no. PVP-40).
3. CTAB Extraction Buffer: 20 m*M* disodium dihydrogen ethylenediamine tetraacetate (Na2EDTA) (Amresco, 0245-500G), 100 m*M* Tris-HCl (Sigma, T-5941). Adjust pH to 8.0 with HCl, 1.4 *M* NaCl, 2.0% (w/v) CTAB (hexadecyl trimethyl-ammonium bromide) (Sigma, H-5882), heat to 60°C to dissolve CTAB and store at 37°C to prevent precipitation. Add in fume hood just before use: 0.2% (v/v) 2-mercaptoethanol (Fisher, 0-3446).
4. 24:1 Chloroform:isoamyl alcohol (J.T. Baker 957-02, Sigma I-1381).
5. 25:24:1 Phenol:chloroform:isoamyl alcohol (Sigma P-2069).
6. Pasteur pipets (Fisher, 13-678-6A) with tips bent into J shape over Bunsen burner.
7. 1X TE Buffer: 10 m*M* Tris-Cl, 1 m*M* EDTA, pH adjusted to 8.0 with NaOH, autoclave.
8. 5 *M* NaCl.
9. 100% Ethanol chilled to –20°C.

3. Methods

3.1. Agrobacterium *Preparation*

1. Streak *Agrobacterium* cultures from previous plates or from –80°C freezer stocks (*see* **Note 3**) onto fresh plates of LB Agar with *Agrobacterium* selection. Incubate for 18–72 h in the dark at 27°C. Store plates at 4°C if they are kept longer than 72 h.
2. Inoculate 3.0 mL of LB broth containing *Agrobacterium* selection with one sterile loop of *Agrobacterium* from the plate prepared in **step 1**. Avoid single colonies; a sample from a streak of thick growth is favorable (*see* **Note 4**).

3. Incubate with rapid (about 200 rpm) shaking overnight in the dark at 27°C. Shake tubes in a slant rack tied to the shaker or propped securely at an angle to allow maximum surface area for gas exchange and maximum agitation.
4. Pour entire contents of this tube (3 mL *Agrobacterium* culture) into one sterile 250 mL Nephelo flask containing 50 mL of AB sucrose minimal medium.
5. Incubate overnight in the dark at 27°C with slower shaking (about 150 rpm).
6. Calculate the concentration of bacteria in a Spectronic-20 spectrophotometer (Bausch & Lomb) set at 620 nm (*see* **Note 5**). The side-arm of the Nephelo flask can be inserted directly into the spectrophotometer after swirling gently to mix, and the 5 mL portion of AB Sucrose minimal media saved earlier should be used for the blank. An absorbance reading of 1.0 is equivalent to 5.0×10^8 cells/mL. A minimum of 10^8 cells/mL (absorbance >0.2, indicates late-log phase growth) is necessary for transformation. If absorbance is less than 0.2, allow cells to grow longer, up to 48 h total incubation. Check concentration before proceeding.
7. Pour cell suspension into sterile 50 mL centrifuge tubes and centrifuge for 8 min at 1900*g*. A thick pellet of bacteria should be visible at the bottom of the tube. Decant supernatant into a waste container to be autoclaved.
8. Resuspend *Agrobacterium* in about 20 mL of induction medium by adding this medium to the centrifuge tube and shaking gently. Pour this culture into a sterile 250 mL standard Erlenmeyer flask and bring volume to 100 mL (2X volume of AB sucrose) with induction medium.
9. Shake slowly (60 rpm) overnight at room temperature. This culture can be stored in the dark up to 5 d at 4°C.
10. Pellet the cells as described in **step 7**.
11. Resuspend *Agrobacterium* in co-cultivation liquid medium to a concentration of 10^9 cells/mL (approximately $^1/_{10}$ of the initial volume). These 10X cultures will be diluted to make co-cultivation solutions when elm leaf squares are ready.

3.2. American Elm Leaf Tissue Preparation

1. If American elm trees are to be grown directly from seed for use in transformation, plant seeds in greenhouse at least 6 wk (*see* **Note 2**) before leaf tissue is needed (*see* **Note 6**).
2. When *Agrobacterium* is ready for co-cultivation (suspended in co-cultivation liquid media) harvest at least 200 healthy American elm leaves. If larger (>4 cm long) leaves are used, fewer leaves need be harvested. Carefully transfer leaves into a sterile 1 L beaker in a sterile hood (*see* **Note 7**).
3. Completely submerge leaves in about 600 mL of the 10% bleach leaf sterilization solution and soak for 7 min with periodic mixing (*see* **Note 8**). From this point on, work only in a sterile laminar flow hood.
4. Decant bleach into a waste beaker, and thoroughly rinse leaves with sterile distilled water three times (or until bubbles from Tween-20 in bleach solution no longer appear) (*see* **Note 9**).
5. Using a sterile scalpel, remove the sides and tip of each leaf, leaving a 0.3–0.5 cm strip on each side of the midvein. On larger (>3 cm) leaves, nick the midvein on the abaxial

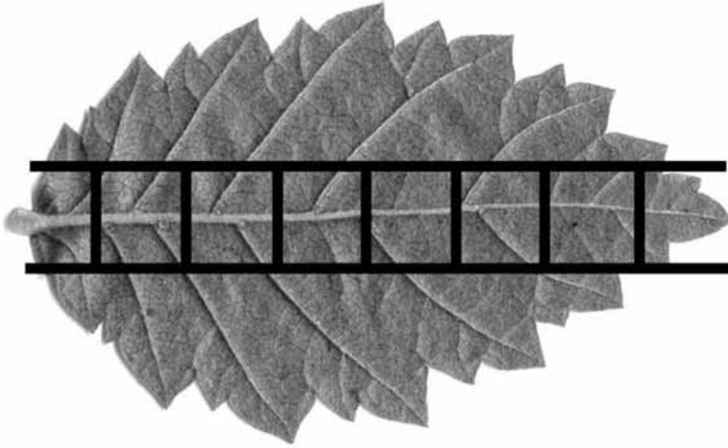

Fig. 1. Cutting instructions for American elm leaf transformation. First make small nicks along entire length of midvein on underside of leaf, but do not cut all the way through the leaf. Then cut along heavy lines, and discard the tip of the leaf and side portions that do not include the midvein.

face (underside) of leaf lightly with the scalpel along its entire length to wound the tissue. Cut strip containing the midvein into pieces about 0.5–1.0 cm long (about 1 cm^2 leaf pieces), (*see* **Fig. 1**). These leaf squares will be used for co-cultivation.

3.3. Agrobacterium *and American Elm Co-cultivation*

1. Dilute 10X co-cultivation stocks from **Subheading 3.1., step 11** with co-cultivation liquid medium to make several tubes of *Agrobacterium* in co-cultivation solution at $>10^8$ cells/mL. For example, add 3 mL of 10X stock to 27 mL of co-cultivation liquid in a sterile 50 mL centrifuge tube.
2. Add freshly cut and nicked leaf squares to the 50 mL co-cultivation tubes. Leaf pieces should be well-soaked and able to move freely within the solution.
3. Add at least 25 leaf pieces to a tube of co-cultivation media without *Agrobacterium* as a nontransformed control.
4. Sonicate tubes in a floating tube rack (leaves in co-cultivation liquid should be submerged in sonicator bath) for 1 min (*see* **Note 10**).
5. Incubate *Agrobacterium* with elm leaf squares for at least 30 min to 1 hr. Liquid co-cultivations of up to 48 h are possible.
6. Transfer leaf pieces from co-cultivation solution to co-cultivation solid medium. From this point forward, leaf squares should be placed face down (veins up) on the solid media.
7. Blot off extra liquid, but don't rinse off leaf pieces yet. Place squares on the medium so they do not touch each other. Approximately 20–25 squares fit well on a 110-mm Petri plate. Seal plates with Parafilm or cellophane sealing tape to prevent desiccation.

8. Co-cultivate leaf squares with *Agrobacterium* on this solid medium at 25°C for 48–72 h in the dark. Co-cultivations of up to 5 d at room temperature or slightly warmer are acceptable.
9. Rinse leaves thoroughly with sterile water and then with sterile water containing 250 μg/mL cefotaxime and 200 μg/mL carbenicillin to remove all visible *Agrobacterium* before culturing the leaf pieces (*see* **Note 11**). Proceed immediately to elm tissue culture.

3.4. American Elm Tissue Culture, Selection, and Screening

1. Arrange 20–25 sterile leaf squares on a 110-mm Petri plate of ESI selection medium. Adaxial leaf faces should be in contact with the media (i.e., face down, midvein facing away from media). Keep leaf pieces at room temperature. Storage under grow lights (90–100 μmol/s/m^2) with a long-day cycle (16-h light/8-h dark) is acceptable. All transfers should be performed in a sterile hood and plates should always be sealed before leaving the hood (*see* **Note 12**).
2. Transfer all leaf pieces to new ESI selection media after 2 wk (*see* **Note 13**).
3. Transfer surviving leaf pieces to new ESI selection media every 2–4 wk until buds develop. Leaf squares that have turned completely brown and flaccid need not be transferred, but any pieces with green spots, turgidity, and/or callus growth should be considered alive and maintained for up to 16 wk.
4. When buds appear (first visible under a dissecting microscope as bumps composed of overlapping green scales), their presence should be noted and they should be stored under grow lights (16-h light/8-h dark, 90–100 μmol/s/m^2), but they should not be transferred immediately to second-stage tissue culture media.
5. When buds grow to 2–3 mm (clearly visible to the naked eye as new leaf buds) they can be carefully separated and transferred to EEL (*see* **Notes 14** and **15**), under the same light conditions as above. Buds can be left on ESI until they form distinct shoots, but once shoots are apparent, they will grow and multiply faster on EEL. A 4 × 4 grid (16 shoots) fits well in one Magenta™ cube containing 45–50 mL of EEL.
6. If your *Agrobacterium* gene construct contains the *gus* gene as the scorable marker, perform a GUS assay on each potentially transgenic line. Aseptically remove a small piece of leaf tissue (3 mm off the tip of a small leaf is sufficient) and incubate in 100 μL of X-gluc in a microcentrifuge tube at 37°C overnight. Treat a nontransformed leaf piece in the same manner as a negative control. Remove X-gluc after 6–24 h, add 500 μL of 100% ethanol to remove chlorophyll and preserve tissue, and note blue coloration indicating expression of the *gus* gene.
7. Subculture shoots into new EEL cubes every 4 wk. Most American elm shoots will have grown taller and clonally produced several small shoots in this time period. Cut off each new shoot and scrape off all callus before transferring to new media (*see* **Note 16**).
8. When shoots get tall (>3 cm) or start to appear brown or woody, they should be transferred to ERM. First, remove the lower leaves and dip the base of each shoot into a sterile solution of 1 m*M* α- naphthaleneacetic acid (NAA) to stimulate root

formation. Then plunge each shoot deep (>1 cm down) into ERM. Watch for formation of thick white roots under or slightly above the surface of the medium.

3.5. Acclimation and Greenhouse care

1. Once roots have formed, the shoot can be aseptically removed from the tall cube of ERM with a scalpel or microspatula. Be sure to include the media directly surrounding the roots when pulling shoots from the cube to avoid breaking roots. Gently carve away most of the media from the base of the shoot without disturbing the roots. Plant the rooted shoot in soaking-wet high-peat-content potting soil mixture (*see* **Note 17**).
2. Keep shoots covered with clear plastic to maintain high humidity (≥80% relative humidity) for at least 7 d, then reduce humidity to allow plants to acclimate to ambient humidity and a cuticle layer to form on the leaves.
3. Re-pot actively growing elm trees frequently into progressively larger pots to prevent them from becoming pot-bound.
4. Several weeks after growth stops and a hard bud forms at the apical meristem, remaining leaves can be removed and the potted tree can be stored at 4°C for >3 mo.
5. After removing trees from this cold treatment, re-pot them into a slightly larger pot and keep them at room temperature under a normal day light cycle. The buds should flush and new growth should form within 1–4 wk. Anecdotal evidence suggests that seed-grown American elm trees will flush sooner and produce new growth after shorter cold treatments than tissue culture-grown trees.

3.6. DNA Extraction From American Elm Leaves (Modified From ref. 30)(see Note 18)

1. Select young, healthy leaves (from new growth, but fully expanded) from your target plant.
2. Measure 1.0 g of leaf tissue and freeze to −80°C.
3. Grind tissue with mortar and pestle with liquid nitrogen until it becomes a very fine powder with no larger chunks visible (cornstarch consistency), and scrape into a 15 mL polypropylene centrifuge tube (do not use tubes made of polystyrene, as it can be dissolved by chloroform used in later steps).
4. Add 6 mL CTAB extraction buffer (with 0.2% 2-mercaptoethanol), invert to mix, add 0.08 g PVP-40, invert until powder is mixed into liquid, and incubate 25 min in a water bath at 60°C.
5. Add 1 volume (6 mL) of 24:1 chloroform:isoamyl alcohol and invert gently until an emulsion forms. Centrifuge for 10 min at 1900*g*.
6. Transfer top (aqueous) phase to a new 15-mL polypropylene centrifuge tube with 5-mL glass pipet (narrow pipet tips may shear genomic DNA). Repeat **steps 5** and **6** until aqueous phase is no longer cloudy (usually two chloroform washes are sufficient).
7. Add 0.1 volume (about 0.5 mL) of *5 M* NaCl, invert gently to mix, and put tubes on ice.
8. Add 2 volumes (about 10 mL) of −20°C 100% EtOH, and invert gently to mix.

9. Store tubes at –20°C overnight to precipitate DNA. (Can be left at –20°C up to 2 wk).
10. Hook out white, cloudy mucus-like DNA with a bent, disposable glass Pasteur pipet (if extracting DNA from more than one sample, use a fresh pipet for each sample) and drop DNA into a 15-mL tube containing 6 mL of 1X TE buffer.
11. Incubate 1 h at room temperature to suspend DNA; heat to 65°C for up to 1 h if necessary until DNA is totally suspended.
12. Repeat **steps 5** and **6** once with 25:24:1 phenol:chloroform:isoamyl alcohol, then twice with 24:1 chloroform:isoamyl alcohol. Additional chloroform:isoamyl alcohol extractions can be performed if necessary to remove phenol residues.
13. Repeat **steps 7–9** to precipitate DNA.
14. Hook out DNA with bent Pasteur pipet as in **step 10,** drop DNA into a 2-mL microcentrifuge tube, and leave caps open to dry DNA thoroughly in a laminar flow hood. Heat to 65°C briefly in a heat block if necessary to dry pellet. Do not overdry (all visible liquid should be gone; pellet should be dried beyond a gummy consistency, but should not be flaky or powdery).
15. Resuspend in 0.5 mL of 1X TE buffer (add up to 1.0 mL additional 1X TE if DNA concentration is very high or if consistency is gummy).
16. Quantify 1:10 dilutions of DNA in spectrophotometer at 260 and 280 nm.

4. Notes

1. 20X AB Salts will tend to form an orange precipitate at 4°C, so stir thoroughly before use.
2. American elm seeds were grown in a standard flat of Fafard Canadian Growing Mix 2 under grow lights (16-h light, 8-h dark, 90–100 µmol/s/m^2, 23°C). Once shoots appear, they should be kept moist by misting until 2–4 true leaves have appeared (4–6 wk after planting). Soil should be kept moist by watering three times/wk or as needed thereafter.
3. We have stored *Agrobacterium* indefinitely at –80°C in a solution of 50% (v/v) glycerol/LB broth.
4. Fifteen Milliliter Falcon tubes with snap-off lids (Falcon, cat. no. 352006, 17 × 100 mm) work well for this step. These tubes are sterile, individually wrapped, and have a relatively wide mouth opening for easy transfers. The lids of these tubes can be snapped shut to seal completely, or left ajar (yet still sterile) for incubation.
5. Make sure you have all ingredients, including antibiotics, in the 5-mL sample blank when setting up the spec-20. Specifically, the red-orange color range of Rifampicin overlaps the 620 nm wavelength read by the spectrophotometer, and would artificially skew results if not present in the blank.
6. American elm trees up to three growing seasons old have been used as leaf explant sources. Leaves should be healthy and young (taken only from new growth), but fully expanded *(24)*.
7. The container used for collecting leaves should not be used for leaf sterilization. Although the bleach solution would sterilize the bottom of the collection container, the top and rim could still harbor contaminants, which could be exposed to the leaves after the bleach is poured out of the container.

8. Make sure all leaf surfaces are soaked and that leaves are not sandwiched together or against the beaker in such a way that will prevent thorough contact with bleach. Leaves can be agitated carefully with a glass rod if necessary (immersion in bleach solution is sufficient sterilization for a clean stirring rod). Do not allow leaves to remain in bleach longer than 12 min.
9. Do not allow leaves to dry completely after rinsing. After the final sterile water rinse, allow about 50 mL of liquid to remain in the bottom of the beaker to keep leaves moist.
10. Sonication creates micro-wounds on the surface of the plant tissue, increasing transformation efficiency ***(31)***. This protocol has been tested with a Branson 2510 Ultrasonic Cleaner, but any standard jewelry sonicator should work as long as it is large enough to accept the bottom half of several 50-mL centrifuge tubes.
11. Rinse each plate of leaf squares (or groups of 2–3 plates leaf squares) in individual sterile 50-mL centrifuge tubes of sterile water and then sterile water with antibiotics. This significantly helps remove the *Agrobacterium* from the cultures.
12. Check plates frequently for bacterial (slimy) or fungal (fuzzy) contamination. If plates become contaminated with *Agrobacterium* at this stage, wash leaf pieces again with water and antibiotics, or try increasing the concentration of cefotaxime in the selection media. Cefotaxime concentrations of up to 500 mg/L have been used with English elm ***(26)***, but note that their media did not include carbenicillin. With any type of contamination, if caught quickly, most pieces on a plate can often be rescued. Remove pieces on the half of the plate farthest from the site of contamination and place them on a new plate of the same medium. Watch this new plate carefully, and if contamination develops, discard the entire plate. With fungal contamination specifically, if the fungus grows long enough to sporulate, it is likely the spores have been spread throughout the plate and it is nearly impossible to rescue any spore-free leaf pieces. If large droplets of condensation appear inside the lids of the Petri plates, they can be stored upside down to prevent flooding until shoots begin to form.
13. Many antibiotics degrade quickly, especially when exposed to light. Therefore, leaving leaf squares on selection media for longer than 4 wk, or transferring leaf squares to old media, may lead to nontransformed shoot development.
14. Multiple buds growing from a single point on one leaf square are most likely all a result of a single transformation event, but this is only confirmed with Southern analysis. Therefore, label and transfer lines from ambiguous buds separately until their origin is confirmed.
15. EEL should include cefotaxime, carbenicillin, and kanamycin (or appropriate selection agent) in the same concentrations as ESI selection media for at least the first 8 wk of stage II culture. This confirms the death of any persistent *Agrobacterium*, which could otherwise interfere with molecular tests for transgene presence. Continued shoot growth in direct contact with the transgene selectable marker serves as further confirmation of transformation and stable T-DNA integration.
16. With consistent transfers to fresh media, the number of shoots can usually be at least doubled every month. If left longer than 4 wk, small newly produced shoots

may die, but larger shoots may eventually produce roots. These rooted shoots can be potted and acclimated to ambient humidity, but they may not immediately produce new growth once potted.

17. If after 4 wk in ERM a shoot is still healthy but has no roots, 20 μL of 1 m*M* NAA can be pipetted directly into the media at the base of the shoot, or the shoot can be removed, dipped in a powdered root inducer containing IBA (such as Hormodin™1), and potted as described in **Subheading 3.5.**, step 1.
18. This DNA extraction protocol has also been used successfully with Chinese chestnut leaves and American chestnut somatic embryos.

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11

Cork Oak Trees (*Quercus suber* L.)

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Summary

A transformation system for selected mature *Quercus suber* L. trees using *Agrobacterium tumefaciens* has been established. Embryos obtained from recurrent proliferating embryogenic masses are inoculated with AGL1 strain harbouring the plasmid pBINUbiGUSint, which carries the *npt*II and *uidA* genes. Evidence of stable transgene integration is obtained by polymerase chain reaction for *npt*II and *uidA* genes, Southern blotting and expression of the *uidA* gene. The transgenic embryos are germinated and successfully transferred to soil.

Key Words: AGL1; *Agrobacterium tumefaciens*; β-glucuronidase; cork oak; *Fagaceae*; kanamycin resistance; pBINUbiGUSint; *Quercus suber*; somatic embryogenesis; tree genetic transformation.

1. Introduction

The cork oak (*Quercus suber* L.) is one of the most characteristic species of the Mediterranean ecosystem. Besides its great ecological value, it produces cork, a natural renewable product of economic interest.

Long reproductive cycles constrain the tree classical improvement. On the other hand, biotechnological approaches are also limited, because oak trees usually are recalcitrant to in vitro techniques. However, biotechnology is less time-consuming and provides the potential to transfer specific traits into selected genotypes without presumably affecting their desirable genetic background. This is of particular importance in woody species, in which many adaptative and economic traits are under nonadditive genetic control, therefore specific genetic make-up has to be transferred to the offspring.

Besides *Q. suber* (*1,2*), only *Castanea dentata* (*3*), *Castanea sativa* (*4–7*), *Nothofagus alpina* (*8*), and *Quercus robur* (*9,10*) have been transformed within

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the family *Fagaceae*. Transgenic studies for juvenile phase shortening, phytoremediation purposes, alterations of the lignin biosynthesis pathway, increased cellulose accumulation and rhizogenesis increase have been accomplished (for review *see* **refs.** ***11*** and ***12***).

A prerequisite for the production of transgenic plants is the availability of a method to regenerate a complete plant from the transformed cell. Cork oak somatic embryo cultures offer an excellent starting point for genetic manipulation: they present low manipulation requirements, high proliferation rates, and plantlets can be obtained from embryogenic lines either initiated from zygotic embryos ***(13,14)*** or induced in leaves from seedlings ***(15,16)***. However desired traits are commonly expressed at maturity, so an effective selection can only be performed on mature trees ***(17)***. Fortunately, repetitive embryogenic lines can also be induced from mature trees of this species ***(18,19)*** opening the possibility to manipulate and clone desired genotypes. In fact, plants have been regenerated from several selected trees ***(20)***.

This protocol describes a simple method to transform *Q. suber* somatic embryos. Embryo clusters are inoculated and selection is carried out on a culture medium with 100 mg/L kanamycin. The complete transformation experiment requires about 10 mo, including transgenic selection, analysis and plant regeneration. Proliferation of putatively transformed embryogenic masses from inoculated explants can be visible at about 3–4 mo after infection. Two to three more months are needed to bulk-up enough material for analysis (polymerase chain reaction [PCR], Southern blot, histochemical assays, etc.) and a minimum of 3 more mo for obtaining plantlets.

The protocol described here allows to obtain up to 30% of transformation efficiency (defined as number of kanamycin resistant independent transformation events from 100 infected embryo clusters—which can consist of one, two, or more merged or joined embryos) with AGL1 *Agrobacterium tumefaciens* strain (pBINUbiGUSint) and the cork oak M10 embryogenic line.

2. Materials

2.1. Plant Materials

Quercus suber L. somatic embryos and clusters from the M10 line (*see* **Note 1**). A protocol to obtain somatic embryos is given in the **Subheading 3.1.,** according to Hernández et al. ***(18–20)***. In that protocol branches up to 4 cm in diameter are used as starting material.

2.2. Bacterial Strain and DNA Construct

Agrobacterium tumefaciens AGL1 strain ***(21)*** harbouring the pBINUbiGUSint binary plasmid ***(22)*** (*see* **Fig. 1**). This plasmid is a derivative of pBIN19 ***(23)***. It

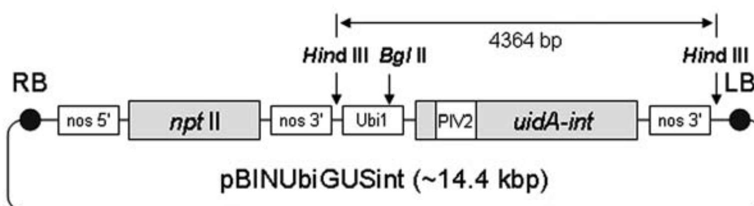

Fig. 1. Brief structure and restriction map of the T-DNA of pBINUbiGUSint (16.1 kb). *Nos5′*, nopaline synthase gene promoter; *Ubi1*, maize polyubiquitin gene promoter; *npt*II, neomycin phosphotransferase II gene; *uidA-int, uidA* gene with the potato PIV2 intron; *nos3′*, polyadenilation signal from the nopaline synthase gene; RB and LB, right and left borders of the T-DNA. Restriction sites for *Hin*d III and *Bgl* II are indicated. The vector backbone is pBIN19 *(23)*.

carries the neomycin phosphotransferase II gene (*npt*II), which confers resistance to the aminoglicoside antibiotic kanamycin, and the β-glucuronidase (GUS) reporter gene interrupted by the PIV2 intron (*uidA-int*). This intron comes from the ST-L1 gene from *Solanum tuberosum* and prevents the gene expression in *Agrobacterium (24,25)*.

2.3. Culture Media

1. Basic medium: Microelements, vitamins, and Fe-ethylene diamine tetraacetic acid (EDTA) from the Murashige and Skoog (MS) medium *(26)*.
2. Preconditioning medium (GMS): Basic medium plus $^{1}/_{2}$ Gamborg (G) macroelements [G (1X) contains 90 mg/L $NaH_2PO_4{\cdot}H_2O$, 30 mg/L Na_2HPO_4, 300 mg/L KCl, 200 mg/L $(NH_4)_2SO_4$, 250 mg/L $MgSO_4{\cdot}7H_2O$, 1000 mg/L KNO_3 and 150 mg/L $CaCl_2{\cdot}2H_2O$] *(27)* and 1% (w/v) sucrose.
3. Primary induction medium (MSSH1): Basic medium plus Schenk and Hildebrandt (SH) macroelements [SH (1X) contains 2500 mg/L KNO_3, 200 mg/L $CaCl_2{\cdot}2H_2O$, 400 mg/L $MgSO_4{\cdot}7H_2O$ and 300 mg/L $NH_4H_2PO_4$] *(28)*, 3% (w/v) sucrose, 10 µ*M* bencyladenine, and 10 µ*M* 1-naphtaleneacetic acid (NAA).
4. Secondary induction medium (MSSH2): Basic medium plus SH macroelements, 3% (w/v) sucrose, 0.5 µ*M* bencyladenine and 0.5 µ*M* 1–NAA.
5. Expression/proliferation medium (MSSH): Basic medium plus SH macroelements and 3% (w/v) sucrose.

The above mentioned media (GMS, MSSH1, MSSH2 and MSSH) were adjusted to pH 5.7 and 0.6% (w/v) agar added prior to autoclaving at 120°C for 20 min.

6. Liquid MSSH medium.
7. YEP medium *(29)*: 10 g/L bacteriological peptone, 5 g/L NaCl, and 10 g/L yeast extract. Adjust pH to 6.9. Add 1.5% (w/v) agar for solid medium and autoclave at 120°C for 20 min. Store indefinitely at room temperature and always check the medium for contamination before use (*see* **Note 2**).

2.4. Antibiotics and Other Stock Solutions

1. Kanamycin: 25 mg/mL stock in water, filter sterilize. Store at −20°C.
2. Rifampicin: 10 mg/mL stock in dimethyl sulfoxide (DMSO) or methanol. Light sensitive. Store at −20°C.
3. Cefotaxim: 50 mg/mL stock in water, filter sterilize. Light and temperature sensitive. Store at −20°C (*see* **Note 3**).
4. 10 m*M* $MgSO_4$: Dissolve 2465 mg of $MgSO_4 \cdot 7H_2O$ in 1L H_2O. Sterilize by autoclaving at 120°C for 20 min and store indefinitely at room temperature.

2.5. Other Supplies

1. 250-mL baby food jars or other appropriate containers for in vitro culture.
2. Sterile filter paper (*see* **Note 4**).
3. Forest containers (180 mL, Arnabat SA, Spain) (*see* **Note 5**).
4. Substrate: pine bark:peat:sand 3:1:1 (v/v/v).
5. REDExtract-*N*-Amp™ Plant PCR Kit from Sigma®(*see* **Note 6**).
6. DNeasy® Plan Mini Kit for DNA isolation from plant tissue (Qiagen®) (*see* **Note 6**).
7. Primers.
   a. *npt*II gene 700 bp sequence: 5′-GAGGCTATTCGGCTATGACTG-3′ (201–222 bp) and 5′-ATCGGGAGCGGCGATACCGTA-3′ (879–900 bp).
   b. *uidA* gene 1199 bp sequence: 5′-GGTGGGAAAGCGCGTTACAAG-3′ (400–420 bp) and 5′-GTTTACGCGTTGCTTCCGCCA-3′ (1579–1599 bp).
   c. *virG* gene 199 bp sequence: 5′-AAGGTGAGCCGTTGAAACAC-3′ (263–283 bp) and reverse 5′-ATCTCAAGCCCATCTTCACG-3′ (443–462 bp).

3. Methods

3.1. Induction of Embryogenesis in Q. suber Leaves (19)

1. Cut branches up to 4 cm in diameter from the selected tree. Remove lateral branches and leaves and culture segments up to 15 cm in length in perlite substrate in appropriate available containers (e.g., 5000 cm^3) at 25 ± 5°C and 80–95% relative humidity in a greenhouse or climatic chamber (*see* **Fig. 2A**). Spray them weekly with fungicides (e.g., 0.5 g/L benomyl and 0.4 g/L captan) to avoid fungal infections.
2. Collect the expanding leaves (0.5–2.5 cm from the base to the apex) with small petioles from the growing epicormic shoots, surface-sterilize them by vigorously hand-shaking in 70% (v/v) ethanol for 30 s followed by commercial bleach (3.5% active chlorine) plus two drops of Tween-20 for 10 min, and finally rinse them with sterile distilled water.
3. Place two surface-sterilized leaves with the abaxial surface on GMS in each Petri dish (6-cm diameter), seal it with Parafilm and culture at 25°C in the dark for 7 d.
4. Transfer the leaves to the MSSH1 and incubate in the dark at 25°C for 30 d.
5. Transfer the leaves to the MSSH2 and incubate with 16-h photoperiod (mixed Sylvania Gro-Lux and Philips cool white fluorescent tubes, 120–180 µmol/m^2/s) at 25°C for 30 d.

6. Transfer the leaves to the MSSH and incubate in the same conditions as previous step, subculturing them every 30 d, until embryo clusters appear (*see* **Fig. 2B**).
7. Isolate embryo clusters on MSSH medium and maintain them in the same culture conditions. Subculture every 20–40 d. The shorter the subculture period, the faster the growth and the lower the number of embryo that reach maturity. These embryos are ready to be transformed.

3.2. Bacterial Strain Culture and Preparation

1. Grow *Agrobacterium* strain on an agar-solidified YEP with 100 mg/L kanamycin and 10 mg/L rifampicin in dark at 28°C for 2- to 3-d (cultures can be stored for up to 1 mo at 4°C).
2. Streak an isolated colony and grow overnight in liquid YEP with 50 mg/L kanamycin, using an orbital shaker with environmental control at 28°C and a shake rate of 250 rpm (*see* **Note 7**).
3. Measure the OD_{600} of the culture and take the appropriate amount to prepare 20 mL of a final OD_{600} of 0.5 (*see* **Note 8**).
4. Pellet the bacteria (3000*g* for 10 min), resuspend the pellet in 10 mL of 10 m*M* $MgSO_4$.
5. Pellet again and resuspend to a final OD_{600} of 0.5 in liquid MSSH medium.

3.3. Inoculation and Coculture of the Embryo Clusters

1. Collect the embryos and embryo clusters (*see* **Fig. 2C**) in liquid MSSH to prevent desiccation (*see* **Note 8**).
2. Inoculate the embryos in 0.5 OD_{600} bacterial-MSSH solution for 20 min with gently shaking (e.g., up to 100 rpm in an orbital shaker) (*see* **Notes 8** and **9**).
3. Blot the embryos on sterile filter paper to eliminate excess of bacteria and transfer them (20–30 embryos by Petri dish) to solid MSSH medium without antibiotics (coculture medium).
4. Coculture for 2 d at 25°C in the dark (*see* **Note 10**).
5. Wash the embryos in liquid MSSH medium with 500 mg/L cefotaxim to eliminate excess of bacteria, blot on sterile filter paper and transfer them (10–15 embryos by baby food jar) to MSSH medium with 100 mg/L kanamycin and 500 mg/L cefotaxime (*see* **Note 11**).

3.4. Selection of Transformed Embryogenic Lines

1. Subculture in MSSH medium with 100 mg/L kanamycin and 500 mg/L cefotaxime (selective medium) every 7–10 d for the first month, and then at 15–20 d intervals at 25 ± 1°C and 16-h photoperiod (*see* **Note 12**).
2. Isolate white proliferating embryogenic masses emerging from initial explants (*see* **Fig. 2D**) and subculture them in selective medium during next three months.
3. Molecular analysis of transgenes (*npt*II and *uidA*) and *Agrobacterium* helper plasmid gene (*virG*) using PCR or histochemical GUS assay, on the putatively transformed

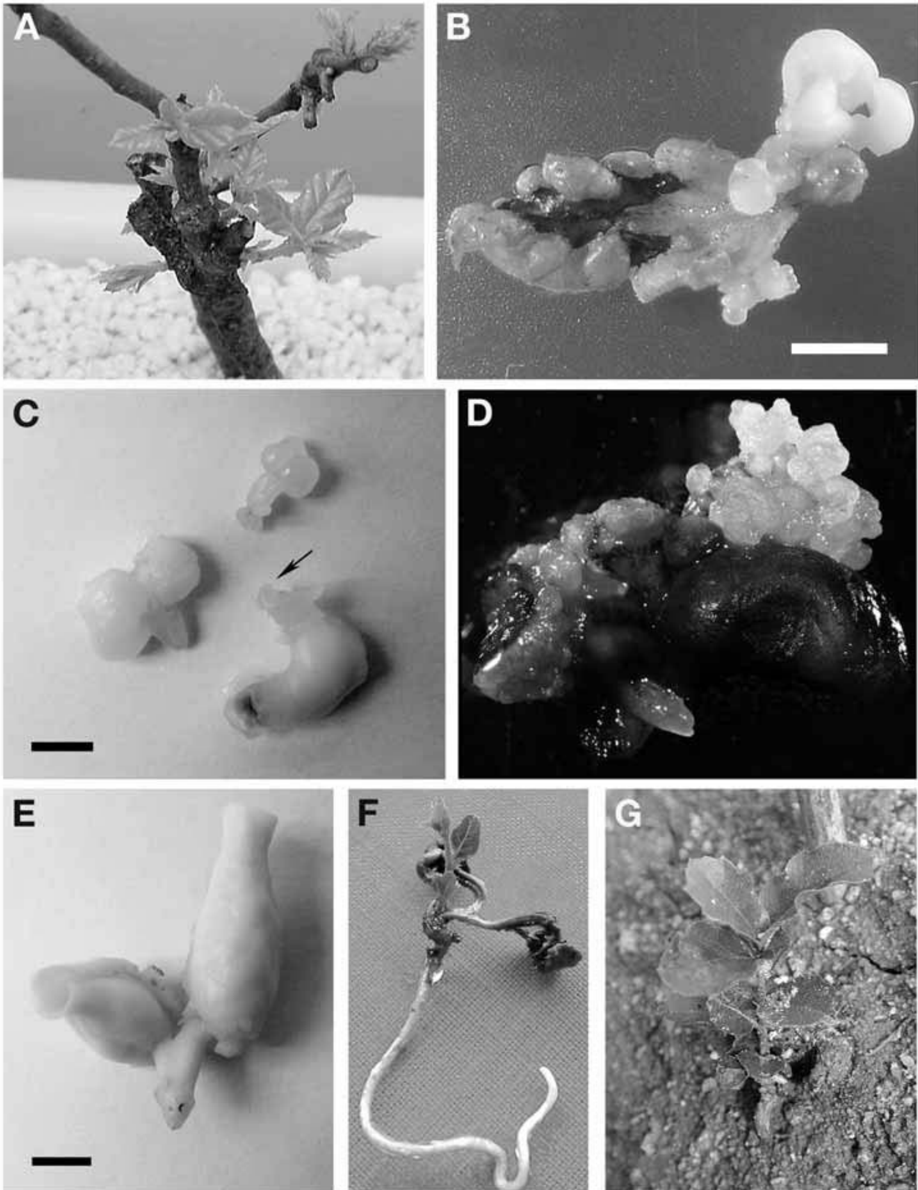

Fig. 2. Stages of the transformation procedure. (**A**) Epicormic shoots with leaves, sprouted in a fragment of branch in perlite substrate. (**B**) Somatic embryogenesis induction on a treated expanding leaf. The somatic embryos formed spontaneously undergo secondary embryogenesis. Bar - 0.4 mm. (**C**) Plant materials to be transformed: *left*, normal embryo; *top*, embryo cluster; *right*, cotyledon with embryogenic proliferation at the embryo axis zone (*arrow*). Bar - 0.4 mm. (**D**) White putatively transformed embryo

lines showing secondary embryogenesis in selective media, can be done at this stage (*see* **Subheadings 3.6.** and **3.7.**).

4. Subculture the kanamycin resistant embryogenic masses on proliferation (MSSH) medium without antibiotics in standard conditions (25°C and 16-h photoperiod) at 20–40 d intervals.
5. Southern blot analysis can be conducted using the secondary embryos of each transgenic clone.

3.5. Plant Regeneration and Conversion (20)

1. Select embryos that spontaneously maturate (white opaque, 15–20 mm in length, average fresh weight of 225 mg and without signs of secondary embryogenesis) (*see* **Fig. 2E**) and transfer them to fresh MSSH medium.
2. Cold culture the selected embryos for 2 mo in the dark at 4°C.
3. Transfer the cultures to 16–h photoperiod at 25°C. About 15 d after they will start germinating (*see* **Fig. 2F**).
4. Once germinated, transfer the embryos to 180 mL forest containers filled with pine bark:peat:sand 3:1:1 (v/v/v) substrate (*see* **Note 13**) and covered with inverted glass beakers, and maintain them in the growth chamber.
5. After 2 mo, remove the beakers for 1 h/d for 1 mo.
6. Move the plants to the nursery and place under shade (*see* **Fig. 2G**).

3.6. Molecular Analyses

For PCR, the DNA can be extracted using either the protocol of REDExtract-N-Amp™ Plant PCR Kit from Sigma® (*see* **Note 6**) or a standard procedure. For Southern analysis, the DNA can be extracted and purifed using the DNeasy® Plan Mini Kit for DNA isolation from plant tissue (Qiagen®) or a standard procedure (*see* **Note 6**).

3.6.1. PCR Amplification

1. Multiplex (for detection of the *uidA* plus *npt*II gene*)* PCR reaction: 4 min at 95°C, 35 cycles of 45 s at 95°C, 45 s at 58°C, and 1 min at 72°C, extension cycle 10 min at 72°C.
2. Conditions for *virG* are: 4 min 95°C, 35 cycles of 45 s at 95°C, 45 s at 56°C, and 1 min at 72°C, extension cycle 10 min at 72°C.

3.6.2. Southern Blot Analysis

Southern blot analyses (*1,30)* must be performed to confirm the stable integration of the transgenes.

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mass, proliferating on a necrotized cluster in presence of kanamycin. (**E**) Appearance of an embryo selected to be cold-treated. Bar - 0.4 mm. (**F**) Germinated somatic embryo. (**G**) Cork oak plantlet growing in field conditions.

3.7. Histochemical (β-glucuronidase) Analysis of Transformants

1. Take embryos proliferating in presence of kanamycin, not exceeding 2–3 mm in size.
2. Incubate in the dark at 37°C for at least 2 h (*see* **Note 14**) in the solution of GUS buffer plus 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) according to Jefferson et al. (*31*).

4. Notes

1. Our current research suggests that genotype of parental tree may have strong influence on transformation efficiency (Álvarez et al, not published).
2. The smaller the agar-media stocks, the lower the time required to melt and thaw them. Therefore 50–200 mL stocks are recommended. Melt the medium in a microwave oven, cool the medium down till about 50°C and then add the appropriate antibiotics.
3. AGL1 *Agrobacterium* strain has a chromosomal resistance to rifampicin and carbenicillin, and the pBINUbiGUSint plasmid confers resistance to kanamycin. Ticarcillin is a β-lactam antibiotic, as carbenicillin is, so it *should not* be used to eliminate AGL1 from the cultures. Cefotaxime is a cephalosporin antibiotic with bacteriostatic properties that works well to eliminate AGL1 from the cultures.
4. Sterilize the filter paper by autoclaving instead of using an oven (100–150°C). After some time in the oven the paper starts to scorch, turns brittle and loses its ability to absorb water. In addition, phenols are produced when scorching and can interfere with the cultures.
5. Square-section forest containers are better than their circle-section counterparts. Roots grow straight to the bottom in the square containers, but in spiral manner along the container wall in the circle ones. The straight growing roots provide high conversion rates (% supervivency of greenhouse plants when transferred to field), but a coiled root system usually cannot support the weight of the aerial part and the plant falls down, resulting in poor conversion rates.
6. The Sigma® kit REDExtract-N-Amp™ Plant PCR is recommended because of its convenience and low cost. It provides DNA extraction buffers and PCRmix (*Taq* polymerase plus buffer, deoxy nucleotide triphosphates [dNTPs], and $MgCl_2$), and no DNA purification is needed. If a classical PCR reaction is preferred, extract DNA with the appropriate method. PCR reaction contains DNA (100 ng), 250 μM of each dNTP, 10 pmol of each primer, *Taq* DNA polymerase, and 2 m*M* $MgCl_2$. For Southern analysis, purification of the DNA is required for the digestion with restriction enzymes to work well. Ten grams of DNA can be easily obtained with the DNeasy® Plan Mini Kit for DNA isolation from plant tissue (Qiagen®) starting from 100 mg of embryos (fresh weight). Do not select too many developed embryos or clusters, because cotyledonar embryos consist of higly-vacuolated cells that can result in a poor DNA extration rate.
7. Cultures take about 16 to 24 h to grow in 20–30 mL of YEP. A preinoculum in 1 mL of medium without antibiotics can be made and added to medium with kanamycin after 4–8 h.

8. Twenty milliliters of bacterial suspension are enough to inoculate 100–150 embryo clusters. 10 mL (2X OD_{600}) of bacterial suspension can be used and mixed with the 10 mL of liquid MSSH employed to gather the embryos when they are collected. So, the phenols released by the embryos could stimulate *vir* genes induction.
9. No wounding is needed other than the act of collecting the embryos with a forceps. Collection can be performed at the same time than preparation of bacteria, saving time. Shaking rates above 100 rpm can result in breaking of the embryos, because the joint between cotyledons and embryo axis is very fragile.
10. Although *vir* gene induction is maximal at approx 25 to 27°C, the pilus of some, but not all, *Agrobacterium* strains is most stable at lower temperatures (approx 18–20°C). Thus, one may consider cocultivating *Agrobacterium* with plant cells at lower temperatures during the initial few days of the transformation process ***(32)***.
11. This step is *strongly* recommended. We have observed that the embryos looked healthier during the subsequent subculture periods when they were washed to eliminate excessive *Agrobacterium*. More importantly, this step reduces the number of explants that suffer bacterial regrowth.
12. Cefotaxime is light- and temperature-sensitive, thereby frequent subculture is recommended to avoid regrowth of bacteria. As commented in ***(19)***, embryos can grow in darkness or in light, so a dim light or darkness culture is a good option to avoid the loss of activity of cefotaxime resulting from light. As the cefotaxime is temperature sensitive, culture conditions above 25°C are not recommended because the antibiotic loses activity faster. Influence of the temperature on the culture of embryos has not been tested, so standard conditions at 25°C are recommended. On the other hand, it is suggested to eliminate dead cotyledons when subculturing embryos, because the bacteria usually regrows on them.
13. Do not wait the radicle to elongate too much before transfer because that reduces the conversion rates.
14. Blue staining appears early and no more than four hours of incubation are needed. A low vacuum can be applied to ease the inflow of the X-Gluc into the embryos. The smaller the embryo samples are, the faster the staining is.

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12

Eucalyptus

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Summary

Using *Eucalyptus camaldulensis* as a model system, we describe here a basic *Agrobacterium*-mediated genetic transformation protocol through organogenesis for the production of transgenic plants. Hypocotyl segments or cotyledon pieces from in vitro seedlings are used as starting materials. The explants are inoculated and cocultivated with a disarmed, binary strain of *A. tumefaciens* CIB542 harboring a mini Ti plasmid, pBI121. A modified Gamborg's B5 medium is used as the basal culture medium throughout stages of co-cultivation, callus induction and shoot regeneration. The incorporation of neomycin phosphotransferase II (*npt*II) and β-glucuronidase (*gus*) genes into the plant nuclear genome are primarily verified by histochemical analysis and polymerase chain reaction (PCR). Modifications of this protocol to use in mature tissues derived from elite trees and other *Eucalyptus* species are also described.

Key Words: *Eucalyptus*; organogenesis; genetic transformation; hypocotyl; cotyledon; *Agrobacterium tumefaciens*; β-glucuronidase (GUS); transgenic trees.

1. Introduction

Eucalyptus is one of the most important commercial planting tree species in the production of raw materials for pulp and paper industry. Genetic engineering of *Eucalyptus* trees has been anxiously anticipated to improve wood quality and forest productivity in a limiting land of plantations. Currently, *Agrobacterium*-mediated transformation still plays the key role in the entire technology of genetic engineering in *Eucalyptus* species because of its efficiency as compared with other transformation methods. In this chapter, a protocol for producing transgenic trees started with juvenile tissues of *E. camaldulensis* by using *Agrobacterium tumefaciens* is described step by step. Using this protocol, putative antibiotic resistant callus pieces are produced from 48 out of 100 initially infected explant materials. Of these 48 callus

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lines, 10 proliferate vigorously and regenerate shoots and plants, which all show positive integration of T-DNA as confirmed by polymerase chain reaction (PCR) or Southern hybridization, indicating a 10% transformation efficiency of the infected explants *(1)*. This protocol has been modified to successfully use in the transformation of various gene constructs using mature tissues of elite clones in *E. camaldulensis*, *E. grandis* as well as *E. grandis* × *E. urophylla*.

2. Materials

2.1. Plant Materials

1. Seeds of *E. camaldulensis*: A seedlot was obtained from Australian Tree Seed Center, Commonwealth Scientific and Industrial Research Organization (CSIRO), Australia. A 10 g weight gives rise to 5000 viable seeds.
2. Shoot tips, lateral buds, or epicomic shoots from elite trees of *E. camaldulensis*, *E. grandis*, *E. grandis* × *E. urophylla* grown in test plantations of Taiwan Forestry Research Institute (TFRI) or in commercial plantations of private pulp and paper companies, and partially collected from elite clones of rooted cuttings maintained in greenhouses of TFRI and North Carolina State University.

2.2. Agrobacterium *Host Strain and Vector Construct*

1. A disarmed binary strain of *A. tumefaciens* CIB542: This strain was derived from EHA101 *(2)* in which the kanamycin resistance gene on the nononcogenic virulence (*vir*) helper plasmid was replaced by a spectinomycin/streptomycin resistance gene.
2. A tumor-inducing (Ti) mini plasmid pBI121: This plasmid contains a selectable marker gene driven by nopaline synthase (NOS) promoter, and a reporter gene driven by cauliflower mosaic virus (CaMV) 35S promoter in a constitutive manner *(3)*. The selectable marker, neomycin phosphotransferase-encoding gene II (*npt*II), confers kanamycin resistance to transformed plant cells. The *uid*A reporter gene encoding β-glucuronidase (GUS) makes transformed plant cells showing blue in the presence of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) (*see* **Notes 1** and **2**).

2.3. Bacterial Medium and Antibiotics

1. Luria–Bertani (LB) medium: 10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl, and 15 g/L Bacto-agar. Adjust pH to 7.0 with 1 *N* NaOH. Appropriate antibiotics should be added to the medium after autoclave.
2. Mannitol-Glutamic Acid: 10 g/L mannitol, 2 g/L L-glutamic acid, 0.5 g/L KH_2PO_4, 0.2 g/L $MgSO_4 \cdot 7H_2O$ and 2 μg/L biotin *(4)*.
3. Mannitol-Glutamic Acid:Luria-Bertani (MGL) medium: a 1:1 (v/v) mixture of mannitol-glutamic acid and LB medium.
4. Stock of 25 mg/mL kanamycin monosulfate dissolved in H_2O and 50 mg/L for working concentration.

5. Stock of 25 mg/mL spectinomycin dihydrochloride hydrate dissolved in H_2O and 50 mg/L for working concentration.
6. Stock of 250 mg/mL streptomycin sulfate dissolved in H_2O and 500 mg/L for working concentration.
7. Stock of 100 mg/mL carbenicillin dissolved in H_2O and 500 mg/L for working concentration.
8. Stock of 100 mg/mL cefotaxime dissolved in H_2O and 500 mg/L for working concentration.
9. All antibiotic stocks should be filter-sterilized and stored at −20°C.
10. Antibiotics such as carbenicillin, cefotaxime and kanamycin are added in the media cooled down to about 45°C.

2.4. Tissue Culture and Plant Growth Medium

1. Sterilization solution: 70% (v/v) ethanol, 1% (v/v) sodium hypochlorite (NaOCl), and sterilized distilled water.
2. Phytohormone preparation: 10 mg/mL of 6-benzylaminopurine (BA), 1-naphthylacetic acid (NAA), and thidiazuron (TDZ) stocks. Phytohormons are dissolved using from 3 to 5 mL of 1*N* NaOH or 1*N* KOH and then final volume is adjusted by slowly adding H_2O. Keep stirring during preparation. These phytohormones can be added in the medium before autoclave.
3. 10X stocks of Murashige and Skoog (MS) medium *(5)*, and Gambogs B5 medium *(6)*: Basal salt mixtures manufactured commercially (M5524 and G5768, Sigma).
4. 1000X MS vitamin stock: 2 g/L glycine, 500 mg/L nicotinic acid, 500 mg/L pyridoxine·HCl and 100 mg/L thiamine·HCl dissolved in H_2O.
5. 1000X B5 vitamin stock: 1 g/L nicotinic acid, 1 g/L pyridoxine·HCl and 10 g/L thiamine·HCl dissolved in H_2O.
6. 100X *myo*-inositol stock: 10 g/L *myo*-inositol dissolved in H_2O. All vitamins can be added in the medium before autoclave.
7. Stock of 100 m*M* acetosyringone dissolved in dimethylsulfoxide (DMSO).
8. MS basal medium *(5)*: MS salts mixture, MS vitamins, *myo*-inositol.
9. B5 basal medium *(6)*: B5 salts mixture, B5 vitamins, *myo*-inositol
10. In vitro germination medium: MS basal medium containing 3% (w/v) sucrose.
11. B5C medium: B5 medium containing 3% (w/v) sucrose, 100 ml/L coconut water, 200 mg/L glutamine, and 100 mg/L casein hydrolysate.
12. Callus/shoot induction medium for hypocotyl and cotyledon explants (CSM_{BA}): B5C medium supplemented with 1 mg/L BA and 3 mg/L NAA.
13. Callus/shoot induction medium for explants derived from field-grown elite trees (CSM_{TDZ}): B5C medium supplemented with 3 mg/L NAA and approx 0.5–1.5 mg/L TDZ (*see* **Note 3**).
14. Selection medium I: CSM_{BA} or CSM_{TDZ} containing 40 mg/L kanamycin, 500 mg/L cefotaxime, and 500 mg/L carbenicillin.
15. Selection medium II: CSM_{BA} or CSM_{TDZ} containing 40 mg/L kanamycin.
16. Shoot proliferation medium (SPM): MS basal medium containing 0.1 mg/L BA.

17. Root induction medium (RIM): Modified MS medium consisting of full strength micro elements and vitamins, half strength of macro elements, and 1 mg/L 3-indolebutyric acid (IBA).
18. All media are adjusted to pH 5.6–5.8 using few drops of 1*N* NaOH or 1*N* KOH and autoclaved at 121°C (15 psi) for 20 mins. Add 7.5 g/L bacto-agar before autoclave.
19. Artificial soil: A mixture of vermiculite, peat moss and perlite (2:2:1 in volume).

2.5. Transgenic Plant Verification

1. GUS histochemical analysis buffer: 1 m*M* 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) in phosphate buffer (100 m*M* primary sodium phosphate, pH 7.0) containing 10 m*M* ethylene diamine tetraacetic acid (EDTA), 0.5 m*M* potassium ferricyanide, 0.5 m*M* potassium ferrocyanide, and 0.1% Triton X-100.
2. DNA extraction buffer: 20 μ*M* Tris-HCl, pH 7.5, 250 μ*M* NaCl, 25 μ*M* EDTA, pH 8.0, 0.5% sodium dodecyl sulfate (SDS).
3. TE buffer: 10 m*M* Tris-HCl, pH 8.0, and 1 m*M* EDTA, pH 8.0.
4. Polymerase chain reaction (PCR) related solutions and buffers.
5. Reaction buffer (10X): 750 m*M* Tris-HCl, pH8.8; 200 m*M* $(NH_4)_2SO_4$; and 0.1% (v/v) Tween-20.
6. 2.5 m*M* each of the deoxy nucleotide triphosphates (dNTPs).
7. Primers: *NPT*II gene forward primer (5′-GAACAAGATGGATTGCACGC-3′) and reverse primer (5′-GAAGAACTCGTCAAGAAGGC-3′).

3. Methods

3.1. Explant Preparation

3.1.1. From Seeds of Eucalyptus camaldulensis

1. Weight 0.1 g of *Eucalyptus* seeds (50 viable seeds) and wrap them with 3 layers of cheesecloth.
2. Immerse the wrapped seeds into 70% ethanol in a 100-mL beaker for 1 min and followed by immersion in 1% NaOCl with ultrasonic treatment for 15 mins (*see* **Note 4**).
3. Move the beaker to a laminar flow hood and rinse the wrapped seeds three times with sterile water.
4. Place and spread surface-sterilized seeds evenly on two Petri plates (100 × 25 mm in size) containing 25 mL germination medium.
5. Seal the plates with parafilm and culture them in an incubator at 25 ± 2°C under approx 140 μE/m^2/sec light intensity with 16-h light/8-h dark cycle.
6. Normally, germination is observed within 5 d after plating. Young seedlings germinated can be transferred into Magenta GA7 boxes or 125-mL Erlenmeyer flasks containing 50 mL of the same germination medium to promote growth.
7. Two days before transformation experiment, isolate hypocotyls or cotyledons from 1-mo-old seedlings germinated in vitro and cut those tissues into small pieces (approx 5-mm length for hypocotyls and 3 × 3 mm for cotyledons).
8. Pre-culture the segments on CSM_{BA} for 2 d at 25°C in the dark.

3.1.2. From Shoots or Buds of E. camaldulensis*,* E. grandis*, and* E. grandis × E. urophylla

1. Surface-sterilize shoot tips, lateral buds, or epicormic shoots by soaking in 70% ethanol for 30 s followed by a rinse in running tap water for 1 h.
2. Move those tissues to a larminar flow hood and soak them into 2% sodium hypochlorite (NaOCl) with ultrasonic treatment (T-28, L&R Manufacturing Co, Kearny, NJ) for 10 min and rinse them thoroughly 3 times with sterilized distilled water.
3. Finally, soak into 100 mg/L filter-sterilized ascorbic acid for 2 h to prevent secretion of phenolic compound causing tissue browning.
4. Culture the surface-sterilized materials on the shoot proliferation medium (SPM) to produce multiple shoot clumps and maintain them at 25 ± 2°C under approx 140 $\mu E/m^2/s$ light intensity with 16-h light/8-h dark cycle. The buds proliferate within 2–3 wk after culturing on the SPM ***(7–9)***.
5. Shoot clumps can be maintained by subculturing small shoot tips onto the SPM biweekly. Excise leaf pieces (approx 3 mm × 3 mm in size) from the in vitro shoot clumps and use them as starting materials for transformation.

3.2. Agrobacterium *Preparation*

1. Three days before transformation experiments, streak a bacterium colony on a LB or MGL plate containing appropriate antibiotics by scrubbing a loop top of bacteria glycerol stock stored in –80°C and incubate the plate at 28°C for 2 d. This *Agrobacterium* plate is then become stock plate and stored up to one month at 4°C for transformation work.
2. Using a loop or toothpick, pick up a single cell colony from the *Agrobacterium* plate and inoculate it into 1 mL LB or MGL liquid broth supplemented with appropriate antibiotics and culture it overnight at 28°C with strong agitation (200 rpm).
3. Pour the overnight-grown bacteria suspension into 25 mL liquid LB or MGL broth in a 125-mL Erlenmeyer flask (*see* **Note 5**). The same antibiotics used for 1-mL bacteria culture should be added in 25-mL bacterial culture. Grow the bacteria at 28°C for 4–6 h with strong agitation (200 rpm) to reach the cell density to 1.0 approx 1.5 OD_{600}.

3.3. Infection and Co-cultivation

1. Pour 25 mL of well grown *Agrobacterium* suspension into a 100 × 25 mm size Petri plate. Immerse the explants into the suspension completely and allow the explants to sit in the inoculum for 10 min with occasional agitation by hand.
2. After inoculation, blot the explants on several layers of sterilized filter paper to remove excess bacteria culture.
3. Transfer the explants onto CSM (CSM_{BA} for tissues from seedlings and CSM_{TDZ} for those from field-grown elite trees) and culture at 25°C in a dark for 2 d for co-cultivation.

3.4. Callus and Shoot Induction

1. After co-cultivation for 2 d, remove the infected segments from the plate and wash them with sterilized distilled water 3 times for 5 min each to remove over-grown bacteria. Five hundred Milligrams per liter cefotaxime and/or 500 mg/L carbenicillin can be added in the water for effective removal of bacteria.
2. Blot the segments onto one layer of sterilized filter paper to remove excess water and transfer them onto selection medium I to select putative transformed cells and prevent proliferation of untransformed cells. For juvenile tissues of *E. camaldulensis*, use selection medium I with 1 mg/L BA. For leaf pieces derived from field-grown elite trees of *E. camaldulensis*, *E. grandis*, and *E. grandis* × *E. urophylla*, use 0.5–1.5 mg/L TDZ.
3. Incubate the plates at 25 ± 2°C under approx 140 µE/m^2/sec light intensity with 16-h light/8-h dark cycle. Subculture the tissues onto fresh selection medium I biweekly.
4. After four rounds of subcultures, transfer the tissues onto fresh selection medium II. Keep subculturing the tissues on selection medium II biweekly until putative transgenic callus appears.
5. Putative transgenic callus can be observed within 30 d and up to 50 d after co-cultivation (*see* **Fig. 1A**). Excise the callus from tissues, culture them onto fresh selection medium II, and maintain the plate at 25 ± 2°C under approx 140 µE/m^2/s light intensity with 16-h light/8-h dark cycle. Ensure you mark the independent callus line.
6. Subculture the excised callus to the same selection medium II biweekly for the callus proliferation and shoot regeneration (*see* **Fig. 1B**).

3.5. Shoot Proliferation and Elongation

1. Shoots are regenerated within about 4 mo after isolation of callus from infected tissues and cultured individually on selection medium II (*see* **Notes 6** and **7**).
2. Shoot proliferation and elongation can be achieved on the same selection medium II. However, to promote shoot proliferation, transfer shoot clumps to shoot proliferation medium when shoot regeneration is visible (*see* **Fig. 2A,B**).
3. Subculture the shoot clumps to fresh shoot proliferation medium biweekly. The cultures are placed in culture room at 25 ± 2°C under approx 140 µE/m^2/s light intensity with 16-h light/8-h dark cycle. Remove the base part of shoot clumps during each subculture.

3.6. Rooting of Shoots and Acclimation of Plantlet

1. Excise the elongated shoots (approx 15 mm in length) from the kanamycin-resistant shoot clumps and transfer them into Magenta GA7 boxes containing 50 mL of rooting medium.
2. Culture the shoots at 25 ± 2 °C under approx 140 µE/m^2/s light intensity with 16-h light/8-h dark cycle for 2–4 wk until primary and secondary roots appear.
3. Although roots emerge within 10 d after culturing on rooting medium, keep culture until the shoot develops more than two roots.

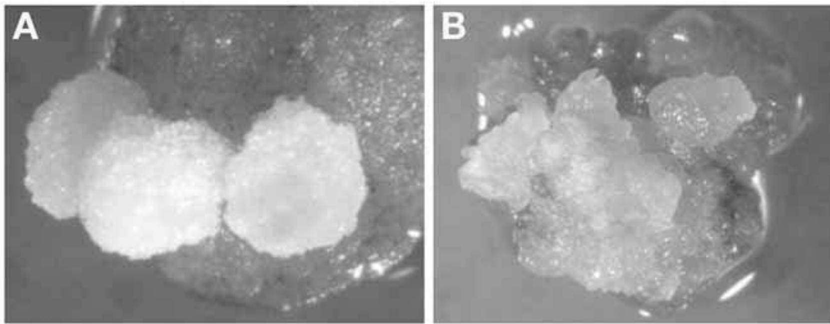

Fig. 1. Putative transgenic callus induction and shoot regeneration. (**A**) Three putative transgenic calli formed from a tissue cultured on selection medium. (**B**) Putative transgenic shoots formation initiated from a callus clump.

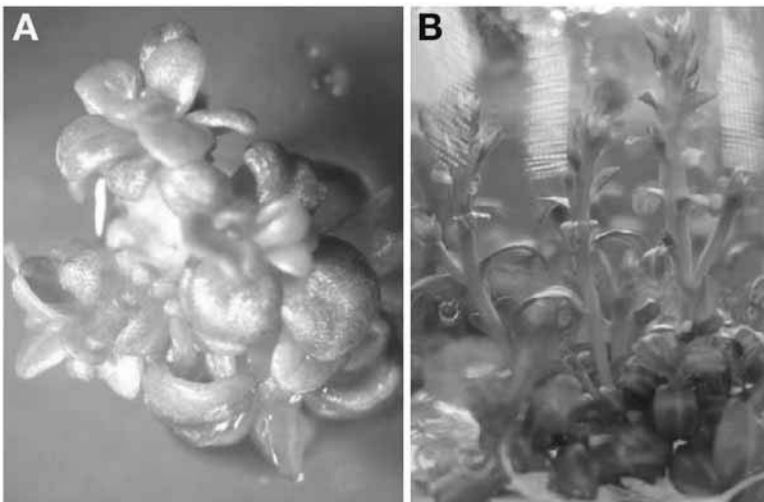

Fig. 2. Shoot proliferation and elongation. **(A)** Multiple shoots proliferated rapidly after transfer onto a shoot proliferation medium. **(B)** Transgenic shoots elongated after subculturing twice. Healthy and well-grown shoots are significant.

4. Carefully take the rooted shoots out from the medium and wash medium away from the roots with running tap water.
5. Transplant the rooted plantlet into a small pot (approx 17 cm in inner diameter) containing artificial soil mixture and cover it with a Magenta GA7 box.
6. Move the pot into a greenhouse at 25 ± 2°C under approx 300 $\mu E/m^2/s$ light intensity with 10-h light/14-h dark cycle and maintain for 1 wk. Water the pot to wet the artificial soil completely, no watering is needed during the first wk of acclimation. Mortality is less than 10% during the process of acclimation.

7. Remove the covered Magenta GA7 box gradually during the period of another week. Water and fertilize the plants regularly.
8. Transgenic plants are cut back at height of 30–100 cm for wood analysis and maintained by trimming regularly at 8-wk intervals.

3.7. GUS Histochemical Analysis

1. To identify *gus* gene integration into a putative transgenic shoot by *gus* gene expression, slice a small part of callus from callus clump or excise a small leaf tissue from shoot.
2. Dip the small callus or leaf tissue into X-Gluc solution and incubate it at 37°C in a dark for 6 h or overnight.
3. After incubation, transfer the callus or leaf tissue into 95% ethanol and incubate overnight to clear the green color from tissues.
4. The intensity of blue color of the tissues indicates *gus* gene integration into the genome and its activity in the cells (*see* **Fig 3A**).

3.8. Genomic DNA Extraction for PCR Analysis

1. Isolate a small leaf tissue (approx 50 mg in fresh weight) from the shoot showing *gus* gene expression.
2. Transfer the leaf tissue into a microcentrifuge tube containing liquid nitrogen and grind it using a small plastic pestle.
3. Add 400 µL of extraction buffer and homogenize for 30 s.
4. Add an equal volume of phenol/chloroform and shake the tube until an emulsion forms.
5. Centrifuge the mixture at 8000*g* for 1 min in a bench top microfuge at room temperature.
6. Use a pipet to transfer the aqueous phase to a new tube. Discard the interface and organic phases.
7. Repeat phenol/chloroform extraction until no protein is visible at the interface of the organic and aqueous phases.
8. Add an equal volume of chloroform, shake, and centrifuge with the same conditions as described above.
9. Transfer the aqueous phase to a new tube.
10. Precipitate the DNA by adding 2 volumes of absolute ethanol and recover the precipitated DNA by centrifugation at 16,000*g* for 5 min at room temperature.
11. Remove the supernatant. Add 1 mL of 70% ethanol and then centrifuge at 16,000*g* for 5 min at room temperature.
12. Remove the supernatant and dry the pellet.
13. Dissolve the pellet in 20 µL TE, pH 8.0.
14. Use *npt*II gene-specific primers to conduct PCR.
15. Perform the PCR reaction as follows:
    a. Reaction mixture: 3 µL template DNA, 5 µL 10X buffer, 0.5 µL 10 m*M* dNTPs, 0.5 µL *npt*II forward primer, 0.5 µL *npt*II reverse primer, 0.5 µL *Taq*-DNA polymerase, and 40 µL double distilled water (ddH$_2$O) to make a total volume of 50 µL.

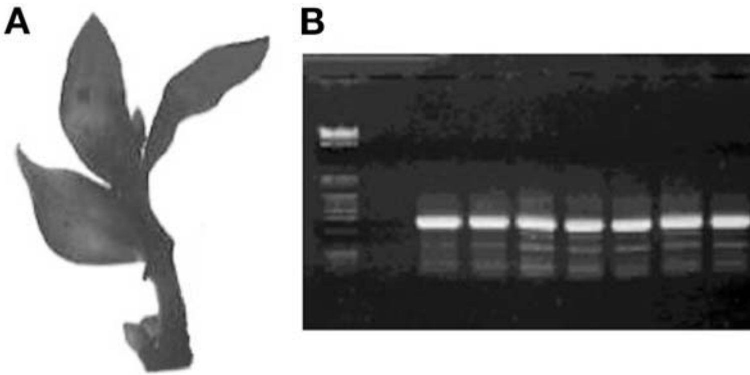

Fig. 3. Primary verification of transgenic tissues. (**A**) Histochemical *gus* expression in transgenic shoots. (**B**) PCR amplification using *npt*II gene-specific primers. A 780-bp band is observed in all transgenic lines. *M* maker; lane 1: untransformed leaf; lane 2–8: different transgenic lines.

b. PCR conditions: 95°C for 120 s, followed by 30 cycles of 94°C for 30 s, 58°C for 20 s, and 72°C for 60 s, and then a final extension at 72°C for 3 min.

16. Electrophorese the PCR product on a 1% agarose gel and visualize the amplicon by staining the gel with ethidium bromide (*see* **Fig. 3B**).

4. Notes

1. This protocol has been successfully applied to introduce various genes into *Eucalyptus* cells including lignin-specific genes such as cinnamate 4-hydroxylase (C4H), 4-coumaric acid:coenzyme A ligase (4CL), coniferaldehyde 5-hydroxylase (CAld5H) and coniferyl alcohol dehydrogenase (CAD), and cold tolerance genes such as C-repeat (CRT)/dehydration element (DRE) Binding Factor (CBF1) and Myb4.
2. The *npt*II gene has been successfully replaced with the hygromycin resistance (*hpt*) gene which confers hygromycin resistance to the transformed cells. To prevent escaped shoot production, hygromycin at 15 mg/L is required for the selection.
3. Use 0.5–1.5 mg/L TDZ instead of BA as cytokinin source for callus induction and shoot regeneration. This is one of the most important factors for successful regeneration of transgenic shoots *(10,11)* (*see also* **Notes 6** and **7**).
4. Healthy seeds are important for germination. Floating seeds in the 70% ethanol are not healthy. Discard them.
5. 100 μ*M* acetosyringone can be added in 25-mL bacteria culture to promote *vir* gene activation. But this is an optional.
6. If no shoots are regenerated with TDZ, zeatin in concentration of 0.5–2 mg/L can be combined with TDZ.
7. Difficulties in shoot regeneration from putative transformed callus and rooting of putative transgenic shoots often result from starting explants which are

unrejuvenated or unhealthy. Using leaf pieces excised from in vitro shoot clumps that have been frequently subcultured as starting explants for transformation experiment may solve the problems in shoot regeneration and rooting of shoots.

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13

Pine (*Pinus radiata*)

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Summary

This chapter describes the transformation of *Pinus radiata* using organogenic cotyledon explants rather than the more common somatic embryogenesis methods for conifers. The advantages of our method are the year round availability of seed and that over 80% of genotypes can be easily regenerated from the mature cotyledon explants. The transformation efficiency (i.e., the number of transformed shoots regenerated from excised cotyledons) is 1.7% and, as with other *Agrobacterium tumefaciens* transformation methods, the majority of transgene integrations are single copy. Critical factors for success are survival of the cotyledons, *Agrobacterium* strain, and selection pressure after cocultivation.

Key Words: *Pinus radiata*; radiata pine; conifer transformation; *Agrobacterium tumefaciens*; organogenic explants; mature seed; transgenic pines.

1. Introduction

Pinus radiata D. Don (radiata pine, Monterey pine) is a major plantation forestry tree in New Zealand, Australia, and Chile. It has the dual purpose of providing wood for timber and for pulp and paper. As with other outcrossing forestry species, genetic engineering offers the opportunity to modify quality traits in a shorter time frame than traditional breeding.

Cotyledon explants from mature *P. radiata* embryos have been successfully transformed with *Agrobacterium tumefaciens*, and transgenic plants have been produced from a range of *P. radiata* genotypes from open-pollinated and control-pollinated seed *(1)*. Regeneration of plantlets from mature zygotic embryos of *P. radiata* is robust and reliable with the majority of genotypes (approx 80%) able to produce many adventitious shoots from the cotyledons *(2–4)*. In addition, mature seed is available year-round. This method overcomes the main disadvantage of direct particle bombardment and *Agrobacterium*-mediated transformation from cultured embryogenic lines of *P. radiata*, which is that very few

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genotypes that can be successfully transformed and regenerated to somatic embryos from such tissue (e.g., *Picea abies* (*5*) and *Pinus radiata* (*6*). *Agrobacterium*-mediated transformation has been reported using embryogenic tissue to produce transgenic somatic embryos (*7*). Successful *Agrobacterium* transformation of organogenic explants has been reported only for loblolly pine (*Pinus taeda*) using mature embryos and shoot apices (*8–10*).

During *Agrobacterium*-mediated transformation of mature *P. radiata* cotyledon explants, there are two factors critical to the production of transgenic plants. The first is survival of the cotyledons after infection with *A. tumefaciens*, as the cotyledons are hypersensitive to *Agrobacterium*. The second factor relates to the efficiency of selection protocols. Plantlets from *Agrobacterium*-infected explants grow slowly and a high proportion of nontransformed shoots must be maintained until detailed molecular analyses can be made. The time from cocultivation with *Agrobacterium* to a plantlet suitable for polymerase chain reaction (PCR) testing is 10–12 mo. The efficiency of production of transformed plants is 1.7% and is recorded as the number of transformed shoots produced divided by the number of initial excised cotyledon explants.

2. Materials

1. LB/MES broth: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 1.066 g/L 2-(*N*-morpholino) ethanesulphonic acid (MES), pH 5.6. Put 50 mL of LB /MES broth in 125-mL Erlenmeyer flasks and autoclave. Stored at room temperature.
2. *Agrobacterium tumefaciens* strain: *Agrobacterium* strain AGL1 (*11*) containing the desired construct. In our study, the typical construct is a derivative of pGA643 vector (*12*). The selectable marker is neomycin phosphotransferase (*npt*II) with the nopaline synthase promoter.
3. *Pinus radiata* seeds: include open pollinated seeds (GF16, GF17, GF19) and control pollinated seed (GF26). Obtained as gifts from Rayonier NZ Ltd, Proseed NZ. Fletcher Challenge Forests Ltd.
4. Hydrogen peroxide (H_2O_2) solution about 30% (BDH Chemicals).
5. Tween-20.
6. Sterile distilled water.
7. 200 mg/mL solution of Timentin® (SmithKline Beecham [Australia] Pty Ltd). Filter sterilized and stored at –20°C.
8. 100 mg/mL solution of kanamycin sulfate. Filter sterilized and stored at –20°C.
9. 100 mg/mL solution streptomycin. Filter sterilized and stored at –20°C.
10. 40 mg/mL and 100 mg/mL solutions of acetosyingone (AS) dissolved in dimethyl sulfoxide (DMSO). Make up in a sterile container and store at –20°C.
11. Glad® wrap plastic food wrap. Each roll cut horizontally into 3-cm sections. Used for sealing Petri plates containing explants.
12. Cocultivation medium: 1/2 LP (modified Lepoivre medium) (*3*): 900 mg/L KNO_3, 200 mg/L NH_4NO_3, 600 mg/L $Ca(NO_3)_2 \cdot 4H_2O$, 135 mg/L $KH_2PO_4 \cdot 2H_2O$, 180 mg/L $MgSO_4 \cdot 7H_2O$, 3.1 mg/L H_3BO_3, 10 mg/L $MnSO_4 \cdot 4\ H_2O$, 4.3 mg/L $ZnSO_4 \cdot 7H_2O$,

0.04 mg/L KI, 0.125 mg/L $Na_2MoO_4 \cdot 2H_2O$, 0.0125 mg/L $CoCl_2 \cdot 6H_2O$, 0.125 mg/L $CuSO_4 \cdot 5H_2O$, 4000 mg/L ethylenediamine tetraacetic acid (EDTA) (FeNa salt), 0.4 mg/L thiamine-HCl, 1000 mg/L myo-inositol, with 5 mg/L benzylaminopurine (BA), 15 mg/L 2,4-dichlorophenoxyacetic acid, 30 g/L sucrose, 8 g/L agar (Bitek™), pH 5.5. Add 20 mg/L acetosynringone (AS) after autoclaving (200 µL of 100 mg/mL stock in DMSO). Poured into 60-mm diameter Petri plates and stored at 4°C.

13. Regeneration medium: 1/2 LP medium with 5 mg/L BA, 30 g/L sucrose, 8 g/L agar (Bitek™), pH 5.8. Add 200 mg/L timentin after autoclaving, pour into 60-mm diameter Petri plates and stored at 4°C (*see* **Note 1**).
14. Selection medium: 1/2 LP medium with 30 g/L sucrose, 8 g/L agar (Bitek™), pH 5.8. Add 200 mg/L timentin and 10 mg/L kanamycin after autoclaving and pour into 60-mm diameter Petri plates, or deep Petri plates, 20-mm height and 90-mm diameter (*see* **Note 2**).
15. Growth medium 1: 1/2 LP medium with 30 g/L sucrose, 8 g/L agar (Bitek™), pH 5.8, with 200 mg/L timentin added after autoclaving and poured into plastic tubs 95-mm diameter and 60-mm high. Store at 4°C (*see* **Note 3**).
16. Growth medium 2: 1/2 LP medium with 30 g/L sucrose, 8 g/L agar (Bitek™), pH 5.8, with 5 g/L activated charcoal. This medium is melted and poured into jars, 9-cm diameter and 10-cm tall, and autoclaved. Store at room temperature (*see* **Note 4**).
17. Pulse medium: 1/2 LP medium with 5 mg/L BA, 30 g/L sucrose, 8 g/L agar (Bitek™), pH 5.8. This medium is melted and poured into jars, 9-cm diameter and 10-cm tall, and autoclaved. Store at room temperature (*see* **Note 5**).
18. Clonex® (Yates New Zealand Ltd) rooting hormone gel containing 3 g/L β-indolylbutyric acid.
19. Potting mix: To 60 L each of peat and bark add 120 L of pumice or perlite, 400 g dolomite lime, 300 g Osmocote®, 75 g superphosfate, 50 g potassium sulfate. Fill plastic pots (15-cm high × 14.5-cm diameter) one-half full with this mix, then top with pumice or perlite.

3. Methods

3.1. Agrobacterium *Preparation*

1. Grow *A. tumefaciens* strain AGL1 culture overnight in 50 mL LB/MES broth with 50 µL streptomycin, 50 µL kanamycin, and 50 µL of 40 mg/mL AS. Put on shaker at 27°C and 100 rpm.
2. The next morning measure the *A. tumefaciens* culture into centrifuge tubes and spin at 3220 g for 6 min. Discard supernatant, add fresh LB broth, and adjust to the required density (0.35–0.40 at OD 550 nm). Add 0.5% dimethyl sulfoxide, (DMSO) (to increase permeability of membranes). Return to shaker (can be at room temperature) as it is now ready to use.

3.2. Explant Preparation

1. Rinse seeds overnight in running water.
2. Surface sterilize seeds with freshly made 10% (v/v) H2O2 plus 1 drop of Tween®20 for 20 min.
3. Rinse twice in sterile distilled water.

3.3. Inoculation, Cocultivation, and Selection

The following steps take place in sterile conditions in a laminar flow hood.

1. Pipette 7 mL of the *Agrobacterium* culture into a sterile 60-mm Petri plate.
2. Dissect embryos from the seeds by cutting seed coat longitudinally with a scalpel, and flicking out the embryo with forceps. Collect embryos onto cocultivation medium.
3. Cut the cotyledons off 20 embryos using a scalpel dipped in *Agrobacterium* culture and transfer to same cultre to cocultivate for one hour.
4. Pipette off *Agrobacterium* culture, plate out cotyledons onto two Petri dishes of cocultivation media (*see* **Fig. 1A**). Place Petri dishes in a growth room at a constant temperature of 22°C. The lighting regime is a 16-h d with cool white fluorescent light at 6 µmol/m^2/s and 8-h night.
5. After 3 d, transfer to regeneration medium, subculture weekly for 2–3 wk. Transfer the regenerating cotyledons to selection medium, subculture weekly for 4 wk, then at intervals of 4 wk for up to 52 wk (*see* **Fig. 1C,D** and **Note 2**).
6. When individual shoots are approx 1-cm tall separate from cotyledon and place in growth medium 1. Maintain the same temperature and day/night times but increase daylight to 20 µmol/m^2/s.
7. When developing shoots are approx 3-cm tall, transfer them to growth medium 2 in jars, and subculture weekly for 6 wk (*see* **Fig. 1E** and **Note 4**).
8. Regenerated shoots are given a 4-wk passage on pulse medium at 3-mo intervals. (*see* **Note 5**).

3.4. Transplant and Glasshouse Care

1. The glasshouse conditions used for the plants was 22°C for a minimum 16-h d and 14°C for an 8-h night.
2. To transfer the plantlets from agar into the glasshouse, individual shoots are removed from agar, dipped in Clonex® and planted in pots.
3. Cover the plantlets with inverted plastic tubs 95-mm in diameter and 60-mm in height. The tubs are then covered with shade cloth for 1 mo.
4. After 6 wk shoots that are not rooted are given a further dip in Clonex®, replanted, and covered with the plastic tubs.
5. When shoots are actively growing and rooted, the tub is gradually removed. One side of the tub is lifted to allow air circulation and gradual reduction of humidity. Three days later the tub is removed completely.
6. Water to keep plants moist but not wet. Fertilize with Plantosan® (Aglukon GmbH & Co., Düsseldorf) (*see* **Fig. 1F**).

3.5. Confirmation of Transformation

DNA is extracted for PCR from in vitro pine shoots.

1. Grind 100 mg fresh weight of *P. radiata* shoot tissue in liquid nitrogen using plastic disposable pestles in Eppendorf tubes.

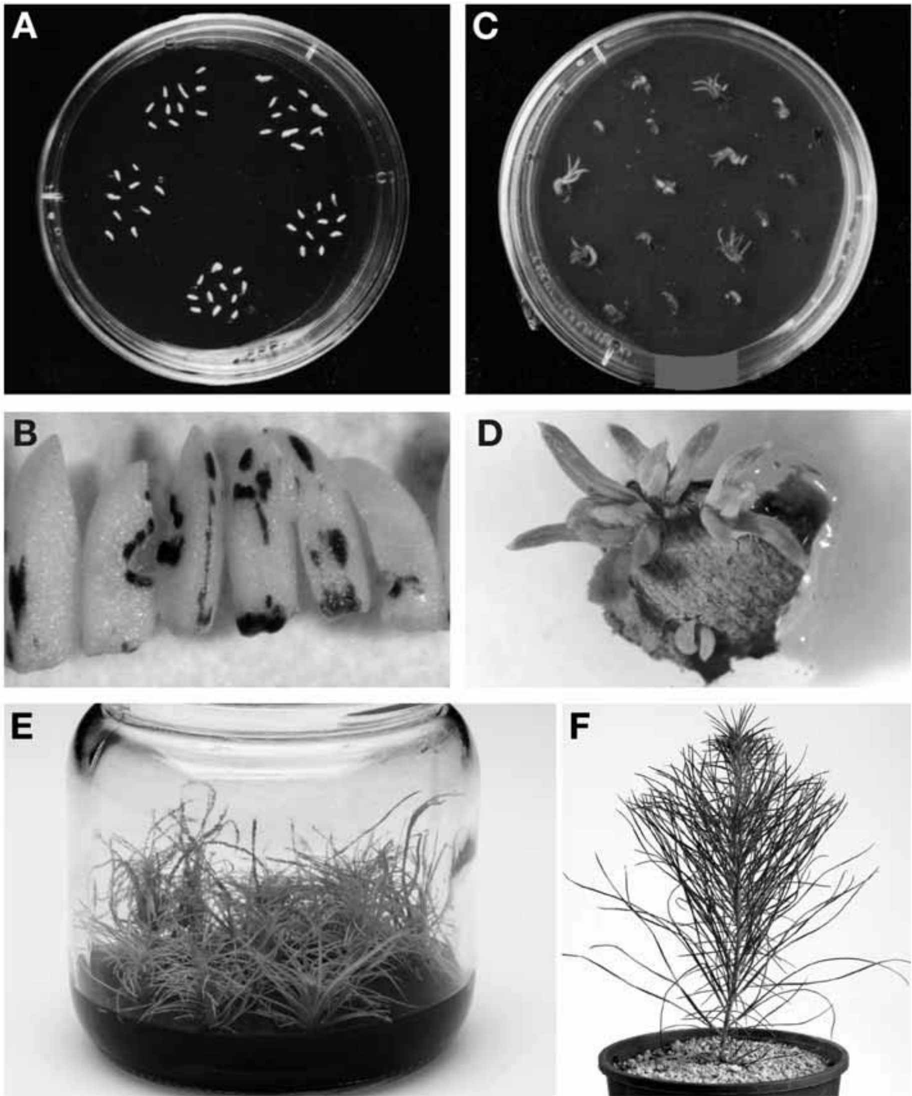

Fig. 1. Steps in *P. radiata* transformation. **(A)** Cotyledons from five embryos after cocultivation. **(B)** Example of *Agrobacterium* infection, 4-d-old cotyledons showing histochemical β-glucuronidase staining (*13*). **(C)** Regenerating cotyledons on selection medium. **(D)** Regeneration of shoots from an individual cotyledon. **(E)** Transgenic clonal shoots of *P. radiata* in Growth medium 2. **(F)** Three month old transgenic *P. radiata* plant in soil.

2. DNA was isolated using the DNeasy Plant Mini kit (Qiagen, Germany) following the manufacturer's instructions.
3. Prepare a 25 μL PCR reaction mix as follows:

   a. 2.5 μL of 10X PCR buffer.
   b. 1.9 μL of 25 m*M* $MgCl_2$.
   c. 2.5 μL of deoxy nucleotide triphosphate (dNTP) mixture (2 m*M*).
   d. 0.25 μL of each primer (20 μM) (*see* **Note 6**).
   e. 0.2 μL *Taq* polymerase (5 U/μL).
   f. 15.4 μL H_2O.
4. To each reaction add 2 μL template DNA.
5. PCR conditions for *npt*II are:
   a. 94°C for 3 min.
   b. 94°C for 40 s.
   c. 62°C for 40 s.
   d. 72°C for 60 s.
   e. Repeat **steps b–d** for 40 cycles.
   f. 72°C for 8 min.
6. For *vir*G PCR, change annealing temperature (step c) to 45°C.
7. Run 12 μL of product on 1% agarose gel.

4. Notes

1. Thiadiazuron (0.5 mg/L) is equally effective as BA in promoting regeneration of transgenic *P. radiata* shoots.
2. Selection of transgenic from nontransgenic shoots is a 'bottleneck' in the process. Although we have successfully produced transgenic plants from selection on kanamycin (10 mg/L and 15 mg/L) and geneticin (5 mg/L and 10 mg/L) we maintained approx 30% of nontransgenic plants. The trade off is between survival in the earlier stages and elimination of nontransformed shoots. The size of Petri dish used depends on the growth of the explant.
3. In the early stages of shoot growth there may be some residual *Agrobacterium* which is controlled by timentin. This step on growth medium 1 allows the shoot to outgrow any *Agrobacterium*. When the shoots are free of contamination they can be transferred to growth medium 2. This step on growth medium 1 without charcoal is necessary as charcoal adsorbs timentin.
4. For growth medium we prefer to use glass jars (9-cm diameter and 10-cm height) as pine shoots grow better and are less likely to become vitrified. Charcoal is necessary for the health of the shoots.
5. Primary transgenic shoots can be maintained for several yr in the growth rooms as long as they are regularly sub-cultured at 6- to 10-wk intervals. One subculture every 3 mo in pulse medium is necessary to maintain multiplication and growth of shoots.
6. Primers for *npt*II are *npt*IIa: 5′-ATGACTGGGCACAACAGACAATCGGCTGCT3′ and *npt* IIb: 5′-CGGGTAGCCAACGCTATGTCCTGATAGCGG-3′; and for *nos*-*npt*II are *nos* fwd 5′-CGC GTT CAA AAG TCG CCT AAG GTC-3′ and for rev = *npt*IIb. For *vir*G use 1 μL of primers - 5′GCG GTA GCC GAC AG3′ and 5′GCG TCA AAG AAA TA3′.

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Poplar (*Populus* spp.)

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Summary

Although species within the genus *Populus* are, in general, easier to transform and regenerate in vitro than most other trees, many poplar species are very recalcitrant. Many protocols that previously have been reported were developed for a specific genotype or species. Thus, it has often been necessary to re-optimize a protocol each time research is initiated with a new genotype. The method presented in this chapter has been effective for a wide variety of poplar genotypes.

Key Words: Aspen; cottonwood; *Populus*; regeneration; transformation.

1. Introduction

Unlike many other trees, species within the genus *Populus* (including aspens and cottonwoods) can be regenerated in vitro via direct organogenesis (as opposed to embryogenesis). Whereas reliable transformation systems have been developed for pure species and hybrids within the section *Populus*, the genotypes in other sections within the genus *Populus* ***(1)*** have been found to be recalcitrant. To date, only a limited number of genotypes in sections *Tacamahaca* and *Aigeros* have been successfully transformed ***(2–7)***. We have developed a protocol that works well with numerous *Populus* genotypes and that is the basis for this chapter ***(8)*** (summarized in **Table 1** and **Fig. 1**). Over the past 10 yr, we have used this protocol to transform 16 different constructs into 13 genotypes (pure species and hybrids) of cottonwood (e.g., *Populus deltoides*, *P. trichocarpa* × *P. deltoides*, *P. deltoides* × *P. nigra*, and *P. nigra* × *P. maximowiczii*) and have produced 705 lines (independent transgenic events). The transformation efficiency (i.e., the number of explants from which a rooted plant is recovered, expressed as a percent of the number of explants that were co-cultivated) varies from 0.4 to 5.0%, depending mostly on the construct being inserted and the genotype

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Table 1
Chronology for the Production of Transgenic Poplar

Subheading	Frame	Description	Materials needed	Time
3.1.1.	A	Poplar pre-growth	Propagation medium	1 mo
3.3.6.	E	Co-culture explants in liquid medium	Overnight *Agrobacterium* culture ($OD_{600\ nm}$ = 0.4 to 0.6)	1 h
3.3.9.	F	Co-cultivation on agar	Callus-induction medium, no antibiotics	2–3 d
3.4.1.		Decontamination	Sterile water and washing solution with 200 mg/L timentin	2 h
3.4.2.		Callus induction	Callus-induction medium with kanamycin and timentin	2–3 wk
3.4.4.	G	Shoot regeneration	Regeneration medium with kanamycin and timentin	4 mo
3.4.6.	H	Shoot elongation	Elongation medium with kanamycin and timentin	1 mo
3.5.1.	I	Rooting shoots	Root-induction medium with kanamycin and timentin	1 mo
		PCR analysis	Transgene-specific PCR primers	1 d
3.5.3.	J, K	Propagation	Propagation medium with kanamycin and timentin	2 mo
3.6.		Transplanting and hardening	Pots, soil, plastic bags	3 wk
		Total time to obtain fully hardened transgenic plants		10–12 mo

Note: "Subheading" refers to the heading in the text under which the procedure is described, "Frame" refers to the appropriate image in **Fig. 1**.

used. We have also generated over 9600 lines by transforming 165 various transgenes into two aspen clones: 717-1B4 (female, *Populus tremula* × *P. alba*) and 353-38 (male, *P. tremula* × *P. tremuloides*). Transformation efficiency for these hybrid aspens has ranged from 5 to 37%, depending on the construct and the strain of *Agrobacterium* used, but the average is generally from about 15 to 20%.

One- to two-month-old in vitro-grown plants are good sources for starting material. Fresh, young shoots from plants grown in a greenhouse can also be used, but those tissues must be surface-sterilized before inoculation with *Agrobacterium*. Explants (leaf discs or stem and petiole segments) are first pre-cultured in the dark to initiate the formation of calli from which shoots will eventually regenerate. Explants from the pre-cultured plant material are then inoculated with an

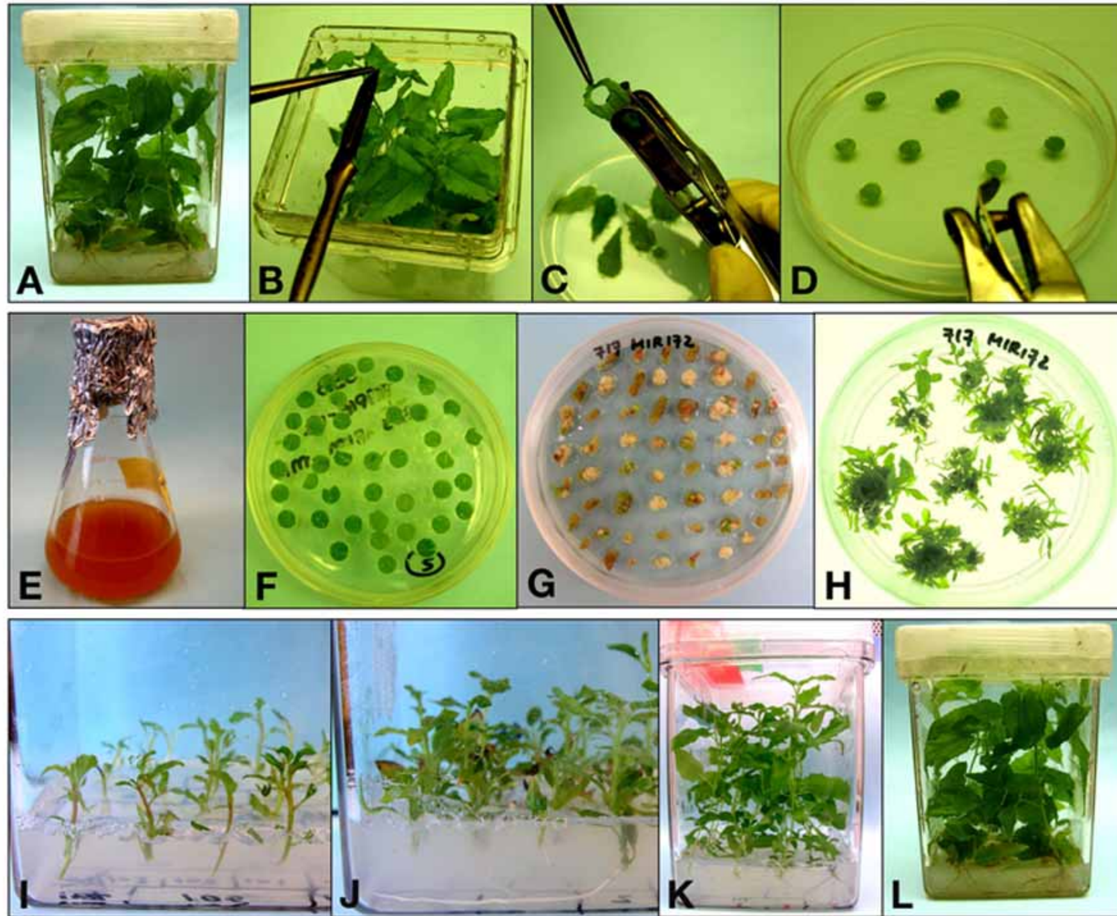

Fig. 1. Steps involved in the production of transgenic poplar.

Agrobacterium cell suspension and co-cultivated before being transferred to a medium that will induce the formation of shoots. Explants bearing nascent shoots are transferred to a medium that induces their elongation before the shoots are excised and then placed on a root-induction medium. Plants that root in the presence of the selection agent, and for which transgene insertion has been verified by polymerase chain reaction (PCR), are transferred to soil and gradually acclimated to ambient conditions before being grown in the greenhouse.

2. Materials

2.1. Plant Materials

Shoot organogenesis can be induced directly on mature leaf explants. Plant preparation is described in **Subheading 3.1**.

2.2. Agrobacterium Strains and Vectors

1. *Agrobacterium* strain: C58/pMp90 (***9***).
2. Genetic constructs: T-DNA is assembled in the pART7 shuttle vector and transferred to the pART27 binary vector (***10***) (*see* **Note 1**). The latter vector has a spectinomycin selectable marker gene outside the T-DNA (to select for the binary vector's presence in its bacterial host) and an *npt*II gene within the T-DNA (to select for transformed plant cells using kanamycin).

2.3. Stock Solutions and Other Supplies

1. Acetosyringone (AS): 50 m*M*.
2. 6-Benzylaminopurine (BA): 0.5 mg/mL (*see* **Note 2**).
3. 2,4-Dichlorophenoxyacetic acid (2,4-D): 1.0 mg/mL.
4. N^6-(2-isopentenyl)adenine (2iP): 5 m*M*.
5. Kanamycin: 25 mg/mL
6. Naphthaleneacetic acid (NAA): 1.0 mg/mL
7. Spectinomycin: 50 mg/mL
8. Thidiazuron (TDZ): 0.5 m*M*
9. Timentin: 100 mg/mL
10. Potting soil: Two parts of perlite mixed with one part peat moss
11. Containers for growing plants ex vitro: "Rose Pots": 5.7 × 8.3 cm (Anderson Die and Manufacturing, Portland, OR).
12. Fertilizer: Peters (Allentown, PA), 20/20/20 (N/P/K), 200 ppm.

2.4. Media

1. Propagation media: 1/2-strength Murashige-Skoog (MS) (***11***), pH 5.8, 0.7% agar (Sigma).
2. Callus-induction medium (CIM)
   a. For cottonwood (CIM1): MS salts, 0.5 μ*M* BA, 0.5 μ*M* zeatin, 5 μ*M* NAA, 5 μ*M* 2,4-D, and 1.28 m*M* 2-morpholinoethanesulfonic acid (MES), adjust to pH 5.8 with 1 *N* NaOH. Add 0.3% Phytoagar and 0.1% Gelrite as solidify agents.

   b. For aspen (CIM2): MS salts, 10 μ*M* NAA, 5 μ*M* 2iP, and 1.28 m*M* MES; adjust to pH 5.8. Add 0.3% Phytoagar and 0.1% Gelrite as solidify agents.
3. Bleach solution: 20% solution of commercial bleach (5.25% sodium hypochlorite) containing 0.1% of Triton X-100.
4. Luria–Bertani (LB) media (*12*): 16 g/L tryptone, 8 g/L yeast extract, and 5 g/L NaCl; adjust to pH 7.0. For solid medium, add 15% agar.
5. Induction medium (IM): 1/2-strength MS salts, MS vitamins, 200 mg/L L-glutamine, 100 mg/L myo-Inositol, 1.28 m*M* MES, and 1.8 g/L D(+)-galactose; adjust to pH 5.0.
6. Rooting medium: 1/2-strength MS containing 0.1 mg/L indole-3-butyric acid (IBA) adjusted to pH 5.8. Add 0.7% agar as solidify agent.
7. Shoot-induction medium (SIM)
   a. For cottonwood (SIM1): MS salts, 10 μ*M* BA, 10 μ*M* zeatin, 1 μ*M* NAA, and 1.28 m*M* MES; adjust to pH 5.8. Add 0.3% Phytoagar and 0.1% Gelrite as solidify agents.
   b. For aspen (SIM2): MS salts, 0.2 μ*M* TDZ, and 1.28 m*M* MES; adjust to pH 5.8. Add 0.3% Phytoagar and 0.1% Gelrite as solidify agents.
8. Shoot-elongation medium (SEM): MS salts, 0.2 mg/L BA, and 1.28 m*M* MES; adjust to pH 5.8. Add 0.3% Phytoagar and 0.1% Gelrite as solidify agents.
9. Washing solution: 1/2-strength MS salts, MS vitamins, 1 μ*M* NAA, 1 μ*M* BA, 1 μ*M* 2iP, 250 mg/L ascorbic acid, and 400 mg/L timentin (*see* **Note 3**); adjust to pH 5.8.

2.5. Growth Conditions

1. Tissue culture growth room: 25°C (continuous) with a 16-h photoperiod; the light is provided by fluorescent tubes (TL70, F25T8/TL735; Philips) at a photon flux density of 45 $\mu E/m^2/s$.
2. Head house: 22–25°C (continuous) with a 24-h photoperiod; the light is provided by fluorescent tubes (cool white, 95 W, F96T12/CW/HO/SS, Sylvania) at a photon flux density of 18 $\mu E/m^2/s$ (*see* **Note 4**).
3. Greenhouse: 25–27°C with a 16-h photoperiod; the light is provided by metal halide bulbs at a photon flux density of 45 $\mu E/m^2/s$.

3. Methods

3.1. Plant Preparation

3.1.1. From In Vitro-Grown Plants

1. Excise shoot tips (3 to 4 leaves) from 1- to 2-mo-old plants growing in Magenta GA-7 boxes containing propagation media (*see* **Fig. 1A,B**).
2. Transfer shoot tips into propagation medium with 0.1 mg/L IBA (*see* **Note 5**).
3. Separate remaining shoot tips into leaves, stems, and petioles (*see* **Note 6**).

3.1.2. From Ex Vitro-Grown Plants

1. Excise shoot tips (8 to 10 cm) from 3- to 6-mo-old greenhouse-grown plants.
2. Gently wash shoot tips in warm, soapy water for 3 to 5 min (*see* **Note 7**), followed by two rinses with sterile, deionized, double distilled (ddH_2O) water.

3. Separate leaves, stems, and petioles.
4. Soak plant parts in 70% ethanol for 1 to 3 min.
5. Gently rock explants in bleach solution for 10 to 12 min.
6. Rinse plant material 3 to 5 times with sterile ddH_2O.

3.2. Explant Preparation and Pre-culture

1. Place explants in sterile ddH_2O in sterile Petri dish (*see* **Note 8**).
2. Use an autoclaved single-hole paper punch in a laminar flow hood to cut discs from leaves (*see* **Fig. 1C**) (*see* **Note 9**).
3. Use a scalpel to cut stems and petiole into segments 5-to 8-mm long (*see* **Note 10**).
4. Wound stem and petiole sections by gently and repeatedly drawing the scalpel blade across their surfaces.
5. Align leaf disks and/or stem explants on CIM and leave Petri dishes in the dark for 1 to 2 d (*see* **Note 11**).

3.3. Inoculation With Agrobacterium and Co-cultivation

1. Streak *Agrobacterium* from frozen glycerol stock (*see* **Note 12**) on solid LB media; grow 1 to 2 d at 28°C.
2. Select an individual colony to inoculate 50 mL LB media containing appropriate antibiotics for the strain of *Agrobacterium* being used (*see* **Fig. 1E**) (*see* **Note 13**).
3. Grow *Agrobacterium* culture overnight on an incubating shaker set at 250 rpm and 28°C. Approximate $OD_{600\ nm}$ at harvest: 1.5 to 2.0.
4. Pellet bacterial cells by spinning for 30 min at 1992*g* (3500 rpm).
5. Dilute cell suspension to $OD_{600\ nm}$ of 0.4 to 0.6 with liquid IM containing 25 μ*M* acetosyringone (AS) (*see* **Note 14**).
6. Place 30 to 40 explants (leaf discs and wounded stem segments) in capped, 50-mL disposable polypropylene, conical centrifuge (Falcon) tube containing 30 to 40 mL of diluted *Agrobacterium* culture.
7. Shake (orbitally) tubes containing *Agrobacterium* suspension and explants at 150 to 200 rpm for 1 h.
8. Decant *Agrobacterium* culture into a flask (*see* **Fig. 1E**) and remove excess *Agrobacterium* from explants by blotting them on sterile paper toweling using sterile forceps (*see* **Note 15**).
9. Align explants on fresh CIM (30–40 explants per plate). Incubate plates (*see* **Fig. 1F**) in the dark for 2 d (*see* **Note 16**).

3.4. Callus Induction and Shoot Regeneration

1. Transfer inoculated explants into Falcon tube; rinse four to five times with 30–40 mL sterile ddH_2O and once with washing solution.
2. Blot explants dry on sterile paper toweling and place onto CIM containing selection agent such as 25 mg/L kanamycin and 200 mg/L timentin (*see* **Note 17**).
3. Incubate explants in the dark for 2–3 wk.
4. Transfer explants to SIM containing 100 mg/L kanamycin and 200 mg/L timentin, culture sealed plates in growth room under lights (*see* **Fig. 1G**).

5. Subculture explants onto fresh SIM every 2–3 wk until shoots form.
6. Transfer explants with multiple small shoots to SEM containing 100 mg/L kanamycin and 200 mg/L timentin (*see* **Fig. 1H**).

3.5. Rooting Regenerants

1. For further selection, excise shoots at 0.5–1.0 cm down from growing tip and place individual shoots on rooting medium containing 25–50 mg/L kanamycin and 100 mg/L timentin. Between 10 and 12 shoots can be placed in each Magenta box (*see* **Fig. 1I**) (*see* **Note 18**).
2. Place Magenta boxes in growth room until roots emergence, usually 1 to 2 wk (*see* **Note 19**).
3. Propagate authentic transgenic plantlets by repeating **steps 1** and **2** of **Subheading 3.5**. (*see* **Fig. 1J,K**).

3.6. Transplanting into Soil

1. Select plants that are 1- to 2-mo post-propagation (about 5 to 6 cm tall).
2. Remove plants from Magenta boxes and rinse away excess agar gently with cold tap water (*see* **Note 20**).
3. Insert plant roots into moist potting mix in a 5.7 × 8.3 cm Rose Pot.
4. Place pots into Zip-Lok® sandwich bags containing about 100 mL of tap water.
5. Transfer plants to growth room, set at 25 ± 1°C and 16-h photoperiod, for about 3 wk.
6. Open bags for progressively longer intervals each day to allow acclimation to ambient conditions (*see* **Note 21**).
7. After 2 to 3 wk, plants can be removed from bags, transferred to greenhouse, and maintained with fertilizer once every 2 to 3 wk (*see* **Note 22**).

4. Notes

1. The binary vector can affect the efficiency with which transgenic plants are recovered. We have found the pART27 backbone to work well. We select for the presence of this binary vector in the bacterial host by using 200 mg/L spectinomycin.
2. Solutions 1–7 are stored at –20°C and filter-sterilized before use; solution 8 is stored at 4°C and no sterilization is needed because it is dissolved in 100% methanol.
3. Ascorbic acid and timentin should be added to the wash solution just before use.
4. In this room we equilibrate our plants (as described in **Subheading 3.6.**) before they are transferred to the greenhouse period.
5. This is a propagation step (so plants will be available for future use). In general, cottonwoods require an exogenous supply of auxin to stimulate root development, whereas aspens do not.
6. Use only fully expanded, healthy leaves.
7. Use a couple drops of standard dishwashing detergent/100 mL water in a 200-mL beaker.
8. This is done to keep the explants from desiccating and browning while being manipulated.
9. Try to include leaf veins when cutting the leaf discs, because a preponderance of shoots arise in the vicinity of the veins ***(13)***.

10. Only use internodes and avoid nodes because the lateral buds frequently give "false-positive" shoots.
11. This step is known as pre-culture and may not be necessary for all poplar genotypes.
12. The stock is made by growing a liquid *Agrobacterium* culture to its stationary phase, mixing an aliquot 1:1 with glycerol in a 2-mL screw-cap freezer vial, and plunging the sealed tube into liquid nitrogen. When stored at −80°C, the cells in these stocks will remain viable for many years.
13. *Agrobacterium* strain C58/pMp90 (*9*) is effective for a wide range of *Populus* genotypes. Other useful strains include: AGL1, EHA105, and LBA4404.
14. Acetosyringone elicits the expression of *Agrobacterium vir*-region genes (*14*). It is best to add the AS immediately before use.
15. Sterilize paper towels by wrapping in aluminum foil and autoclaving for 20 min at 121°C.
16. This step is commonly referred to as co-cultivation. Do not allow severe *Agrobacterium* overgrowth. Normally, 2 to 3 d of co-cultivation is sufficient; after 4 to 7 d, the bacterium will overgrow the explant, making it difficult to control.
17. The selection agent used will depend on the selectable marker present on the T-DNA of your binary vector. Only those plant cells containing the T-DNA bearing the selectable marker gene will survive in the presence of the selection agent to which the marker provides resistance. The *npt*II gene is the most commonly used selectable marker; it imparts resistance to kanamycin. The concentration of kanamycin typically used with poplar is 50 mg/L. The optimum concentration of kanamycin or any other selection agent (e.g., an herbicide) will need to be determined empirically for the particular genotype of *Populus* with which you are working. The goal is to minimize the number of "escapes" (nontransformed cells that regenerate into plants) but avoid overwhelming the transgenic cells with too much antibiotic. The timentin is incorporated into the medium to kill the *Agrobacterium*.
18. It is important to record the date, the plate, and the explant from which the shoot was taken, and not to select shoots that are connected to the same callus mass. The goal is to have shoots that regenerated from cells representing independent transformation events (also known as "line").
19. Shoots that root in kanamycin-containing media are likely to be transformed. However, it is necessary to verify the presence of the transgene in the regenerated plants via PCR, and eliminate the possibility of escapes.
20. Media removal should be thorough in order to avoid fungal or bacterial contamination during plant acclimation.
21. The bag should be opened for 10 to 20 min on the first day. During this time, the plants must be checked frequently. If the plants begin to wilt, mist them with water and close the bag immediately. On successive days, the bags can be left open for progressively longer intervals (try doubling the time the bag is left open each day). It is very important not to stress the plants to the point that they wilt.
22. Plants can be maintained in vitro, in the greenhouse, or in the field. If the latter is done, a permit will need to be obtained from the USDA Plant and Animal Health Inspection Service (APHIS; *http://www.aphis.usda.gov/brs/index.html*).

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15

Rubber Tree (*Hevea brasiliensis* Muell. Arg)

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Summary

Rubber tree (*Hevea brasiliensis* Muell. Arg.) is an important industrial crop for natural rubber production. At present, more than 9.5 million hectares in about 40 countries are devoted to rubber tree cultivation with a production about 6.5 million tons of dry rubber each year. The world supply of natural rubber is barely keeping up with a global demand for 12 million tons of natural rubber in 2020. Tapping panel dryness (TPD) is a complex physiological syndrome widely found in rubber tree plantations, which causes severe yield and crop losses in natural rubber producing countries. Currently, there is no effective prevention or treatment for this serious malady. As it is a perennial tree crop, the integration of specific desired traits through conventional breeding is both time-consuming and labour-intensive. Genetic transformation with conventional breeding is certainly a more promising tool for incorporation of agronomically important genes that could improve existing *Hevea* genotype. This chapter provides an *Agrobacterium*-mediated transformation protocol for rubber tree using immature anther-derived calli as initial explants. We have applied this protocol to generate genetically engineered plants from a high yielding Indian clone RRII 105 of *Hevea brasiliensis* (Hb). Calli were co-cultured with *Agrobacterium tumefaciens* harboring a plasmid vector containing the Hb superoxide dismutase (SOD) gene and the reporter gene used was β-glucuronidase (GUS) gene (*uidA*). The selectable marker gene used was neomycin phosphotransferase (*npt*II) and kanamycin was used as selection agent. We found that a suitable transformation protocol for *Hevea* consists of a 3-d co-cultivation with *Agrobacterium* in the presence of 20 m*M* acetosyringone, 15 m*M* betaine HCl, and 11.55 m*M* proline followed by selection on medium containing 300 mg/L kanamycin. Transformed calli surviving on medium containing 300 mg/L kanamycin showed a strong GUS-positive reaction. Upon subsequent subculture into fresh media, we obtained somatic embryogenesis and germinated plantlets, which were found to be GUS positive. The integration of *uidA*, *npt*II, and HbSOD transgenes into *Hevea* genome was confirmed by polymerase chain reaction (PCR) as well as Southern blot analysis.

Key Words: Rubber tree; *Hevea brasiliensis;* genetic transformation; *Agrobacterium*

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tumefaciens; polymeruse chain reaction (PCR) amplification; Southern blot hybridization; superoxide dismutase gene (SOD).

1. Introduction

Hevea brasiliensis, the para rubber tree, is the most important source of natural rubber; other rubber bearing plants are of minor importance. About 90% of natural rubber is produced in Southeast Asia. Natural rubber is considered a vital raw material by developed countries and is valued for its high-performance characteristics. Synthetic rubber, derived from petroleum, is not as elastic or resiliant and does not have the heat transfer properties of natural rubber. Although synthetic rubber is often blended with natural rubber, various products such as airplane tires require natural rubber *(1)*. In the rubber tree, latex is produced in highly specialized cells called laticifers. When the bark of the rubber tree is tapped, the cytoplasmic contents of these laticifers are expelled in the form of latex. Latex is a milky substance, which upon coagulation and further processing yields natural rubber.

The rubber tree (*Hevea brasiliensis*, $2n = 36$) is a perennial tropical crop, which belongs to the genus *Hevea* and the family Euphorbiaceae. The genus *Hevea* encompasses ten species, all originating from the Amazon region *(2)* and all strongly outcrossing and monoecious. Most of the natural rubber exploited in the world originates from this single species. As in other crops, rubber production is influenced by various plant physiological conditions and pathogenic diseases. However, latex production still faces serious economic losses over the world as a result of tapping panel dryness (TPD), or brown bast a syndrome characterized by an abnormal reduction of latex flow. During tapping the latex from a healthy rubber tree flows through 3–4 h by turgour pressure inside the bark tissue. Once the TPD occurs, the tapping incision is partly or entirely blocked and the amount of latex production is significantly decreased or stops completely. The incidence of TPD occurs in 12 to 50% of rubber trees in almost every rubber producing country.

Genetic improvement of *Hevea* is very slow and time consuming as in many other perennial species. The major limitations are the very narrow genetic base, nonsynchronous flowering, low fruit set, long gestation period, heterozygous nature, and absence of fully reliable early selection parameters. Genetic engineering is a powerful method for crop improvement when specific genetic changes need to be made in a short time period without loss of genetic integrity. Although the transfer of DNA into plant cells via *Agrobacterium* and other methods is now routine for many plant species, the coupling of transformation with the selection of transformed cells and regeneration of transgenic plants is still difficult in many economically important tree species including *Hevea*. TPD is considered to be a serious physiological disorder caused by oxidative stress in

rubber tree that are frequently tapped for natural rubber in the form of latex. In *Hevea* an increase in the level of the free radical scavenging by the incorporation of a superoxide dismutase (SOD) gene could enhance stress tolerance in plant cells against oxidative stress. The genetic manipulation of *Hevea* has been made in the recent past. Transgenic plants have been developed with marker genes via *Agrobacterium* as well as particle bombardment methods ***(3,4)***, however, the transformation efficiency was generally low. Montoro et al. ***(5,6)*** studied the response of calcium on *Agrobacterium*-mediated gene transfer in *Hevea* friable calli but did not obtain transgenic plants. So far no such agronomically important gene has been successfully transformed into *Hevea*. Recently our group developed transgenic *Hevea* plants by incorporating the SOD gene ***(7,8)***.

The transformation protocol presented in this chapter was established in the author's laboratory in combination with review of the literature in tree transformation. For this *Agrobacterium*-mediated transformation, we used a constitutive version of the *Hevea brasiliensis*-SOD gene (HbSOD) by infecting 2-mo-old anther derived calli as initial explant. The β-glucuronidase gene (*uidA*) was used for screening and neomycin phosphotransferase gene (*npt*II) was used for selection of the transformed calli. The transformation efficiency was 4%. The overall scheme employed for this study is outlined in the flowchart in Fig. 1. In this chapter we describe the steps involved in rubber transformation protocol and isolation of DNA as templates.

2. Materials

2.1. Plant Materials

Two-mo-old calli derived from immature anther or inflorescence (Indian clone RRII 105) (*see* **Fig. 2A**).

2.2. Agrobacterium tumefaciens *Strain and Gene Construct*

We used the binary vector pDU96.2144 ***(9)*** which contains *uidA* as a reporter gene and *npt*II as selectable marker gene plus the HbSOD gene under the control of constitutive promoter (CaMV 35S).

Binary vector is inserted into disarmed *Agrobacterium tumefaciens* strain EHA101 to create functional vector for transformation experiments.

2.3. Stock Solutions

1. 20X Modified Murashige and Skoog (MS) major salts stock solution ***(7)***:
   a. MS1: NH_4NO_3, 20.0 g/L; KNO_3, 32.0 g/L; $MgSO_4$ (anhy), 3.60 g/L; $CaCl_2$ (anhy), 6.66 g/L; and KH_2PO_4, 3.4 g/L.
   b. MS2 and MS3: NH_4NO_3, 10.0 g/L; KNO_3, 16.0 g/L; $MgSO_4$ (anhy), 1.80 g/L; $CaCl_2$ (anhy), 3.32 g/L; and KH_2PO_4, 1.70 g/L.

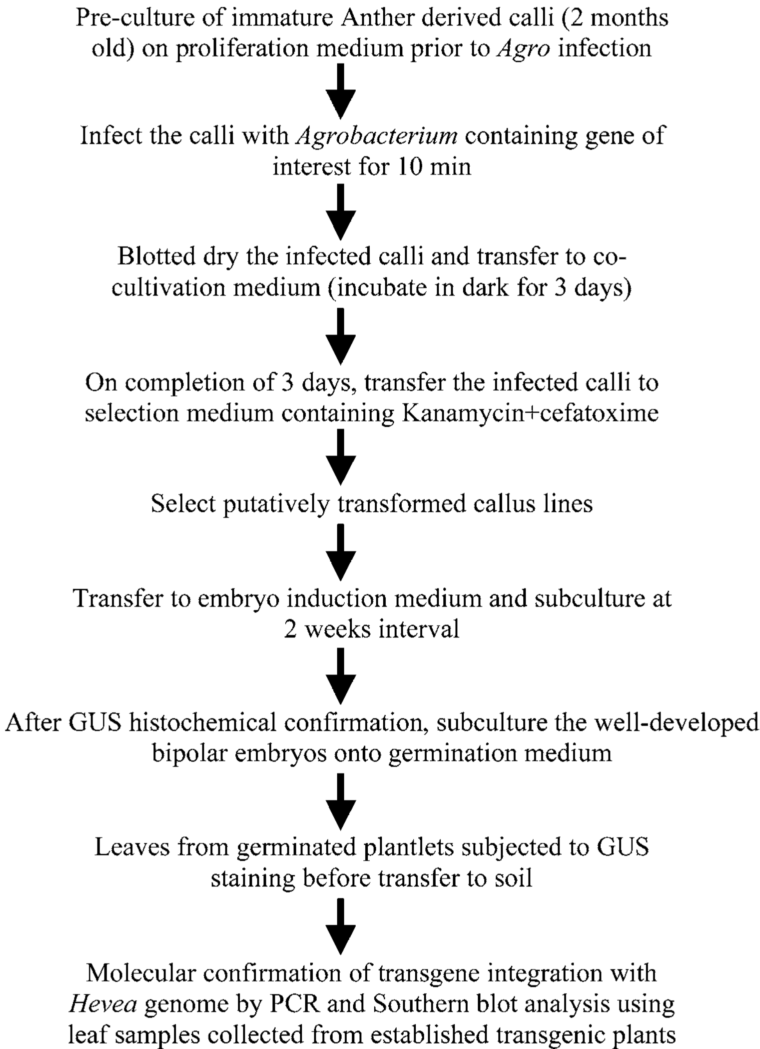

Fig. 1. Flow chart of rubber transformation protocol.

2. 100X MS minor stock solution: H_3BO_4, 620 mg/L; $CoCl_2{\cdot}6H_2O$, 2.5 mg/L; $CuSO_2{\cdot}5H_2O$, 2.5 mg/L; $MnSO_4{\cdot}4H_2O$, 1.69 g/L; $NaMoO_4{\cdot}4H_2O$, 25 mg/L; KI, 83 mg/L; and $ZnSO_4{\cdot}7H_2O$, 860 mg/L.
3. 100X Iron Stock: Na_2EDTA, 3.72 g/L and $FeSO_4{\cdot}7H_2O$, 2.78 g/L.
4. 100X B5 vitamin stock solution: Myo-inositol, 10 g/L; nicotinic acid, 100 mg/L; pyridoxine HCl, 100 mg/L; and thiamine HCl, 1.0 g/L.
5. Auxins (Sigma, 1.0 mg/mL for each stock solution): 2,4-Dichlorophenoxy acetic acid (2,4-D), indole-3-acetic acid (IAA), 1-naphthalene acetic acid (NAA). To prepare

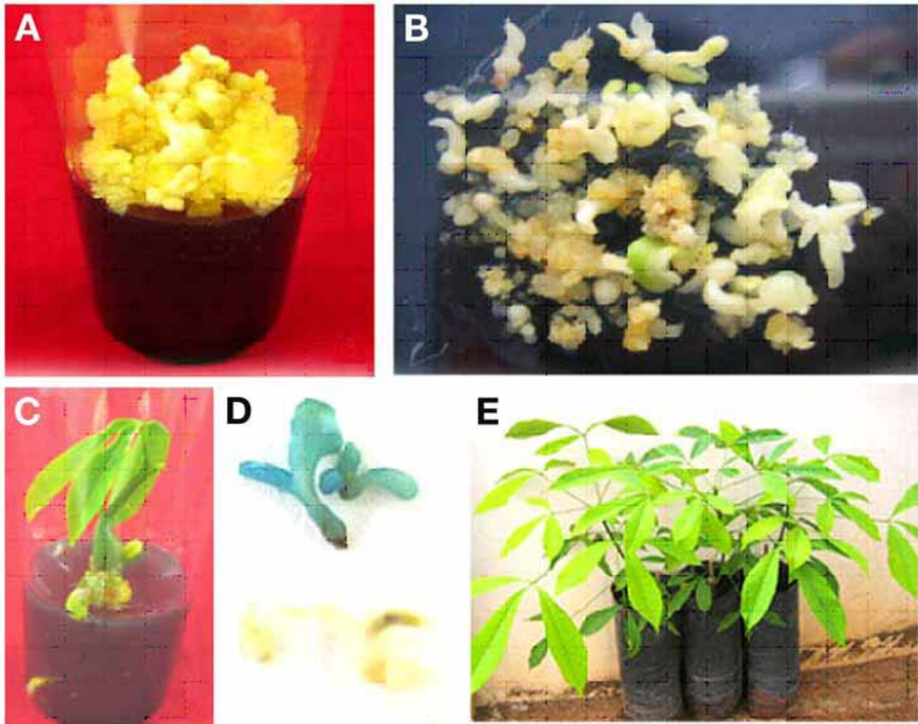

Fig. 2. Development of transgenic plants and molecular confirmation of the presence of transgenes in rubber tree (*Hevea brasiliensis*). **(A)** Callus proliferation, **(B)** Different stages of somatic embryos, **(C)** Germinated plantlet, **(D)** Histochemical GUS assay (transgenic embryos showing blue colour), **(E)** Plants established in polybags.

individual solution, dissolve each powder in 50 µL of 1*M* KOH and then add sterile distilled water to 1 mL volume. Store at 4°C for up to 3 mo.

6. Cytokinins (Sigma, 1.0 mg/mL for each stock solution): 6-Benzylaminopurine (BA), kinetin (KIN). To prepare individual solution, dissolve the chemical in 50 µL of 1*M* HCl then make up to 1 mL volume with sterile distilled water. Store at 0°C indefinitely.
7. Abscisic acid (ABA) (1.0 mg/mL, Sigma): Dissolve the powder in 100 µL of methanol make final volume with sterile water and store at 4°C for upto 3 mo.
8. Gibberellic acid (GA_3) (1.0 mg/mL, Sigma): Dissolve the chemical in ethanol (100%) and store at 4°C for up to 3 mo.
9. 100X B5 vitamins (Sigma): To make a 50 mL stock solution, dissolve 500 mg myoinositol, 50 mg thiamine HCl, 50 mg nicotinic acid, and 5 mg pyridoxine HCl in sterile distilled water and store at 4°C for up to 3 mo.
10. 20 mg/mL gentamycin stock solution (Sigma): Dissolve the 20 mg powder in the vial with 1 mL sterile distilled water. Store at −20°C for up to 3 mo.

11. 100 mg/mL kanamycin stock solution: Weigh 1 g of kanamycin monosulfate (Sigma), place in a beaker, and add 8 mL of sterile distilled water. Allow kanamycin to dissolve completely. Make the final volume to 10 mL with sterile water then filter sterilize using 42 μ*M* pore size membrane and divide into 1-mL aliquots. Store at –20°C for up to 6 mo.
12. 500 mg/mL Cefotaxime (Sigma) stock solution: Dissolve the chemical in sterile distilled water then filter sterilize and aliquot into 1 mL. Store at –20°C for up to 3 mo.
13. 100 m*M* Acetosyringone (Sigma) stock solution: Dissolve the chemical in sterile distilled water, filter sterilize, and aliquot into 1-mL Eppendorf tubes. Store at –20°C for up to 3 mo.
14. 100 m*M* Betaine HCl (Sigma) stock solution: Dissolve the chemical in sterile distilled water and then filter sterilize and aliquot into 1-mL Eppendorf tubes. Store at –20°C for up to 3 mo.
15. 50 m*M* Proline (Sigma) stock solution: Dissolve the chemical in sterile distilled water and then filter sterilize and aliquot into 1-mL Eppendorf tubes. Store at –20°C for up to 3 mo.
16. 1.0 mg/mL Spermidine (Sigma) stock solution: Dissolve the chemical in sterile distilled water and then filter sterilize and aliquot into 1-mL Eppedondorf tubes. Store at –20°C for up to 6 mo.

2.4. Media

1. AELB medium: 10 g/L Bacto-tryptone (Sigma), 5 g/L yeast extract (Sigma), and 15 g/L Bacto Agar (Sigma), pH 7.2. Autoclave, cool to 50°C, and add 50 mg/L kanamycin and 20 mg/L gentamycin. Mix well and pour into sterile 90-mm Petri plates.
2. Callus induction and proliferation medium (MS-1): To make 1 L, add 50 mL of 20X modified MS major salt stock solution (*7*), 10 mL each of 100X MS minor stock solution, 100X iron stock solution, and 100X B5 vitamin stock solution, 5% sucrose (w/v), 2.0 mg/L 2,4-D, 0.5 mg/L KIN, 1.0 mg/L NAA, adjust to pH 5.7 with 0.1*M* KOH and add 0.25% (w/v) phytagel as solidifying agent then autoclave at 121°C for 20 min.
3. Somatic embryo induction and maturation medium (MS-2): To make 1L, add 50 mL of modified MS major salt stock solution, 10 mL each of 100X MS minor stock solution, 100X iron stock solution, and 100X B5 vitamin stock solution, 5% sucrose (w/v), 0.1 mg/L 2,4-D, 0.3 mg/L NAA, 0.3 mg/L BA, 2.0 mg/L GA_3, 0.1 mg/L ABA, 200 mg/L casein hydrolysate, 150 mg/L malt extract, 100 mg/L banana powder, 10% coconut water (w/v), 2 mg/L spermidine, adjust to pH 5.7 with 0.1 *M* KOH and add 0.4% phytagel as solidifying agent then autoclave at 121°C for 20 min.
4. Embryo germination and plantlet regeneration medium (MS-3): To prepare 1 L, add 50 mL of modified MS major salt stock solution, 10 mL each of 100X MS minor stock solution, 100X iron stock solution, and 100X B5 vitamin stock solution, 0.3 mg/L GA_3, 0.5 mg/L KIN, 0.1 mg/L IAA, 0.5 mg/L BA, adjust to pH 5.7 with 0.1*M* KOH and add 0.2% (w/v) phytagel as solidifying agent then autoclave at 121°C for 20 min.
5. Co-cultivation medium: To prepare 1 L, take the same MS-1 media components as described above and autoclave, cool to 50°C then add 20 μ*M* acetosyringone, 15 μ*M* betaine-HCl, 11.55 μ*M* proline.

6. Selection medium: To prepare 1 L, take the same MS-1 media components as described above and autoclave, cool to 50°C then add 300 mg/L kanamycin and 500 mg/L cefotaxime, mix thoroughly and dispense 25 mL into 90-mm sterile petri dishes.

2.5. Other Reagents, Solutions and Supplies

1. Surface disinfection solution I: 0.5% (w/v) sodium hypochloride, 0.1% (v/v) Tween-20.
2. Surface disinfection solution II: 0.1% (w/v) mercuric chloride.
3. Sterile Whatman filter paper No. 3 (SD fine, India).
4. Sterile distilled water.
5. Sterile metal spatulas with spoon on one end (Fisher, USA).
6. Soil mix: Soil rite (Himedia, India), sand in 1:1 ratio and autoclave. Fill in polythene bags (10 × 20 cm W × H).
7. X-Gluc solution (Sigma): 0.1 μ*M* phosphate buffer, pH 7.0, 10 m*M* ethylene diamine tetraacetic acid (EDTA), 0.5 m*M* potassium ferricyanide, 0.5 m*M* potassium ferrocyanide, 0.1% (v/v) Triton X-100, and 2 m*M* X-Gluc (5 bromo-4 chloro-3-indolyl -β-D glucuronide).
8. Cetyltrimethyl ammonium bromide (CTAB) extraction buffer: 2%(w/v) CTAB (hexadecyltrimethyl ammonium bromide), 1.4 *M* NaCl, 20 m*M* EDTA, pH 8.0, 100 m*M* Tris-HCl, pH 8.0, 1% (w/v) polyvinyl polypyrrolidone (PVPP), and 1% (v/v) β-mercaptoethanol. Store at room temperature.
9. Phenol/chloroform/isoamyl alcohol (25:24:1): Mix 25 parts phenol (equilibrate in 100 m*M* Tris-Cl, pH 8.0) with 24 parts chloroform and 1 part isoamyl alcohol. Add 8-hydroxy quinoline to 0.1% (w/v) Store in aliquots at –20°C ≤ 6 mo.
10. Chloroform/isoamyl alcohol (24:1).
11. RNase A (DNase free, 10 mg/mL): Dissolve RNase A in 10 m*M* Tris-Cl, pH 7.5 and 15 m*M* NaCl; boil for 10 min and allow to cool to room temperature. Store aliquots at –20°C to prevent microbial growth.
12. Proteinase K: 10 mg/mL in H_2O. Store at –20°C.
13. 100% isopropyl alcohol, ice-cold.
14. 70% (v/v) ethanol, ice-cold.
15. TE buffer; 10 m*M* Tris-HCl, pH 8.0, 1 m*M* EDTA, pH 8.0.
16. Organic solvent-resistant Oak-ridge centrifuge tube (Tarson, India).

3. Methods

3.1. Callus Initiation and Pre-culture

The following tissue culture protocol is modifications of Jayashree et al. ***(10)***.

1. Collect flower buds (0.5–1.0 cm size) and surface disinfect in 100 mL of surface disinfection solution I for 5 min followed by thorough washing with sterile distilled water 5 times.
2. Surface sterilizes the flower buds with 100 mL of surface disinfection solution II for 3 min followed by 5 rinses in sterile distilled water.

3. Dissect out the immature anthers and place approx 10 anthers/tube on MS-1 medium (*see* **Note 1**). Incubate cultures at 26°C in the dark and subculture at 4-wk intervals into fresh medium of the same formulation.
4. Pre-culture the 2-mo-old callus (*see* **Note 2**) on fresh MS-1 medium prior to infection with *Agrobacterium* (*see* **Notes 3** and **4**).
5. Place the culture in controlled environment at 26 ± 2°C for 2 d. Photoperiod of 16-/8-h light/dark cycle with cool-white florescent light (60 µmol/m^2/s).

3.2. Agrobacterium *Culture Preparation*

1. Streak *Agrobacterium* using loop from a glycerol stock onto AELB medium supplemented with 20 mg/L gentamycin and 50 mg/L kanamycin. Incubate at 28°C for 2 d.
2. Transfer a single well-grown *Agrobacterium* colony from plate into a 25 mL liquid AELB medium with appropriate antibiotics.
3. Keep 25 mL cultures onto an incubator shaker (28°C) for 24 h with agitation (250 rpm).
4. Grow cells overnight until an A_{600} of 0.5; adjust the bacterial cell density to 5×10^8 cells/mL and use for transformation.

3.3. Agrobacterium *Infection and Co-cultivation*

1. Transfer the precultured calli into the *Agrobacterium* suspension and immerse for 10 min.
2. Collect the infected calli using sterile spatula and blot dry on sterile Whatman No. 3 filter paper to remove excess of bacterial suspension.
3. Transfer the calli on coculture medium (10 callus pieces/plate) and incubate them under 26 ± 2°C in the dark for a period of 3 d (*see* **Note 5**).

3.4. Selection of Transformed Callus

1. After 3 d of coculture, subculture the calli into selection medium. Seal plates with parafilm and maintain at 26 ± 2°C in the dark. Subculture every 2 wk for a period of 2 mo (*see* **Note 6**).
2. Omit cefotaxime from the selection medium and screen for putatively transformed callus lines in the presence of kanamycin (*see* **Notes 7–10**).
3. Select 8-wk-old kanamycin resistant callus lines for embryo induction.

3.5. Somatic Embryogenesis and Plant Regeneration

1. Transfer the putatively transformed calli growing on selection medium and culture on MS-2 medium for embryo formation.
2. Subculture the kanamycin resistant embryogenic callus lines to fresh MS-2 medium at 2-wk intervals. Transfer embryos into fresh MS-2 medium for maturation and incubate for 4 wk with 16-h photoperiod.
3. Select mature bipolar embryos (*see* **Fig. 2B**) and subject them to histochemical GUS assay (*see* **Fig. 2D**) (*see* **Note 11**).
4. Place mature bipolar embryos containing cotyledons on fresh MS-3 medium for germination and keep at 16-h photoperiod.

5. Mature embryos germinate into plantlets in the MS-3 regeneration medium within 2 wk of culture (*see* **Fig. 2C**) (*see* **Note 12**).
6. Transfer the plantlets (>5-cm long) formed in the medium into sterile half-strength MS liquid medium for 2 wk. Establish these plants into small polybags containing autoclaved soil mix, cover with a plastic bag and keep under controlled conditions. Then make a small hole in the plastic bag for aeration (*see* **Notes 13 and 14**).
7. Remove the cover at the end of 2 wk and transfer plants to large polybags, move to glass house for hardening (*see* **Notes 15 and 16**). Initially keep the plants under shade to avoid direct sunlight.
8. After 2 wk, shift the acclimatized rubber transgenic plants to the net house under normal field condition with sunlight (*see* **Fig. 2E**).
9. Budgraft the well-established transgenic plants to normal root-stock grown in net house for multiplication.
10. Collect the leaves of the acclimatized transgenic plants for histochemical staining and molecular analysis such as GUS histochemical assay, PCR, and Southern blot hybridization.

3.6. Isolation of Plant Genomic DNA, PCR, and Southern Blot Analysis

The following is the modifications of Dellaporta et al. ***(11)***.

1. Label the 50-mL centrifuge tubes, collect 2 g of leaves from transformed plants as well as untransformed control plant and place them in the labeled tube (*see* **Notes 17 and 18**).
2. Rinse the leaves with cold sterile water three times in beaker, blot dry on Whatman No. 1 paper.
3. Place the leaves in a mortar, add liquid nitrogen, and grind to a fine powder with a pestle (*see* **Note 19**).
4. Transfer the frozen powder to 50-mL Oak ridge centrifuge tube, which is an organic solvent-resistant tube.
5. Immediately add 20-mL warm CTAB extraction buffer to the pulverized fine powder and gently mix to wet thoroughly. Incubate for 30 min at 65°C with frequent mixing (*see* **Note 20**).
6. Extract the homogenate with an equal volume of 25:24:1 phenol/chloroform/Isoamyl alcohol. Mix well by gentle inversion. Spin for 10 min at 10,000*g*.
7. Transfer about 18 mL of aqueous fraction (upper phase) by using pipetman to fresh centrifuge tube. Be careful not to take the interphase which can decrease your DNA quality (*see* **Note 21**).
8. Add 10 μL each of 10 mg/mL proteinase K and 10 mg/mL RNase A to the homogenate mix well by inversion several times. Incubate the tubes in the 37°C for 20 min, with occasional agitation.
9. Add an equal volume of 24:1 chloroform/Isoamyl alcohol. Mix well by gentle inversion. Spin for 10 min at 10,000*g*, at 4°C. Collect top aqueous phase.
10. Precipitate the DNA by adding exactly 0.6 volume of isopropanol (ice-cold) and mix well. If precipitate is visible, proceed to step 11. If not, place mixture 30 min at −20°C.

11. Spin for 15 min at 14,000*g* at 4°C. Discard the supernatant without disturbing the pellet.
12. Wash the DNA pellet with ice-cold 70% (v/v) ethanol, spin for 2 min at 10,000*g*. Discard the supernatant and air dry for 10 min (*see* **Note 22**).
13. Resuspend the DNA pellet in a minimal volume of TE buffer (100–500 μL/gram of starting tissue material).
14. Take 2 μL DNA and analyze the undigested DNA on 1% agarose gel to determine the integrity and quality (*see* **Note 23**).
15. Amplification of transgenes from genomic DNA by PCR using gene specific primers *(7)*.
16. Confirm the integration and presence of the HbSOD transgene in the putatively transgenic plants through Southern blot analysis *(7)*.

4. Notes

1. Immature anthers at diploid stage are very essential for 100% callus initiation.
2. We found that the 2-mo-old callus is ideal for genetic transformation in *Hevea*.
3. Pre-culture of calli in the proliferation medium is necessary to increase the transformation frequency.
4. Wound with sterile dissection needle enhances the transformation efficiency.
5. A 3-d co-cultivation period is optimal for transformation experiments and cocultivation beyond 3 d results in bacterial over-growth that will destroy the callus.
6. It is found that subculture is to be performed at 2-wk intervals into fresh medium of the same formulations to avoid drying of tissues.
7. We observed that 300 mg/L kanamycin is the optimal concentration to select transformed cell lines in *Hevea*.
8. If the kanamycin concentration in the medium was increased beyond 350 mg/L, a decrease in transformation frequency was noticed.
9. In our experiment, profuse growth of nonstransgenic calli was observed from 0 to 200 mg/L kanamycin, which indicated that kanamycin concentrations up to 200 mg/L were ineffective for selecting transformed cell lines.
10. We used 20 m*M* acetosyringone in the co-cultivation medium to enhance the transformation frequency.
11. Eight-wk-old kanamycin-resistant mature bipolar embryos were subjected to GUS assay before transfer to embryo germination medium. It is very important to confirm the transgene integration events before the development of plants.
12. Media supplemented with ABA, polyamines, and organic supplements and with optimal agar concentrations favored embryogenesis and the regeneration of transgenic plants.
13. The leaves from germinated plantlets were subjected to GUS expression assays, and all were found to be GUS-positive.
14. Initially, we kept germinated plantlets in liquid half-strength hormone free MS medium for 2 wk; this step is essential before transfer to soil rite.
15. We used plastic bags to cover the plants. It is important to cover each plant immediately after transfer to soil rite to prevent wilting.

16. If the plantlets established in soil rite are kept in direct sunlight, heat will build up under the plastic cover and dry the plant.
17. GUS positive plantlets were used for molecular confirmation of the presence of *uidA, npt*II, and HbSOD transgenes by PCR using specific primers.
18. The integrity of the nucleic acids will be improved by maintaining harvested tissues cold. We have observed that modified CTAB method give DNA of better quality.
19. In order to get better results, the following precautions should be followed during the preparation of reagents for DNA isolation and during isolation, PCR, and Southern blot hybridization procedures wear gloves at all times. Use highest quality molecular biology grade reagents. Use sterile, disposable plasticwares if possible.
20. The success of this DNA isolation procedure hinges on the ability to gently disrupt cellular integrity while maintaining DNA in an intact, high-molecular-weight form. Thus, mixing of the tissue sample and phenol should be performed by gentle inversion, which minimizes shearing forces on the DNA.
21. It is important that aqueous phase must be collected without disturbing the protein interphase to get good quality DNA.
22. The DNA pellet may be air dried for 10 min.
23. To ensure the DNA isolated is of high quality, it is advisable that DNA samples are examined by running in a 1% (w/v) agarose gel before Southern analysis are performed.

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IV

Tropic Plants

16

Banana (*Musa* sp.)

Juan B. Pérez Hernández, Serge Remy, Rony Swennen, and László Sági

Summary

Cultivated bananas are vegetatively propagating herbs, which are difficult to breed because of widespread male and female sterility. As a complementary gene transfer method in banana, the described *Agrobacterium* protocol relies on highly regenerable embryogenic cell cultures. Embryogenic cells are infected and co-cultivated in the presence of acetosyringone with *Agrobacterium tumefaciens* harboring a binary plasmid vector to obtain a mixed population of transformed and untransformed plant cells. Transformed plant cells are promoted to grow for 2 to 3 mo on a cell colony induction medium containing the antibiotics geneticin or hygromycin as selective agents, while agrobacteria are counterselected by timentin. The whole procedure, including plant regeneration, takes approx 6 mo and results in an average frequency of 25 to 50 independent transgenic plants per plate, which equals 50 mg of embryogenic cells. This method has been applied to a wide range of cultivars and to generate large populations of transgenic colonies and plants for tagging genes and promoters in banana.

Key Words: *Agrobacterium tumefaciens*; banana; embryogenic cell suspension; genetic transformation; *Musa* sp.; transgenic plant.

1. Introduction

In terms of production and consumption, bananas and plantains (*Musa* spp.) are the most important fruit crop on Earth, and provide a staple food for about 400 million people in developing countries throughout the tropics. Almost 90% of the annual production (ca. 100 million metric tons) *(1)* is used locally for cooking, baking, boiling, and brewing as well as consumed fresh from a wide range of cultivars grown by subsistence farmers. The remaining 10% has a global value of about $5 billion on the international trade market, which provides for the main exporting countries a significant source of their foreign exchange.

Banana improvement with classical methods is hampered by high sterility levels and triploidy in most edible cultivars. Genetic transformation, therefore,

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may assist banana breeding programs by providing transgenic lines with improved traits. Though an efficient method for direct gene transfer via particle bombardment of embryogenic cell suspensions has already been described in banana *(2)*, progress has also been made in *Agrobacterium*-mediated transformation. Banana was generally regarded as recalcitrant for *Agrobacterium*-mediated transformation. Similarly to grasses, wild-type *Agrobacterium tumefaciens* was indeed not capable of tumor formation in various banana tissue explants (J.B. Pérez Hernández and L. Sági, unpublished). However, the compatibility of various banana tissues with *Agrobacterium* was first demonstrated during chemotaxis and attachment *(3)*, the very early phases of the interaction. Further, several papers reported the generation of transgenic plants after cocultivation of meristematic in vitro tissues *(4)* or embryogenic cell cultures initiated from the male flower *(5,6)* with *A. tumefaciens*.

Here we describe a protocol for *Agrobacterium*-mediated gene transfer to embryogenic cell suspensions generated from proliferating meristematic cultures *(7)*. The procedure consists of the following main steps: (1) growth and induction of *Agrobacterium* cultures, (2) preparation, infection and cocultivation of embryogenic cell suspensions, and (3) selection and regeneration of transgenic plants. Because transformation is targeted to embryogenic cells that are not easy to quantify, we define transformation efficiency as the number of antibiotic-resistant independent events (as regenerated transgenic plants) from a plate containing 200 µL of embryogenic cell suspension at 33% settled cell volume, which equals approximately 50 mg of fresh weight cells. The transformation efficiency ranges, according to this definition, between 10 to 100 independent events depending on the regeneration capacity of a particular cell suspension line. In our experience, 25 to 50 independent transgenic events per plate are a realistic average. This efficiency allowed us to generate large transgenic populations tagged with novel promoter and gene trapping constructs *(8)*.

2. Materials

2.1. Plant Material

Embryogenic cell suspensions of the cultivar ‘Three Hand Planty’ initiated from proliferating meristematic cultures *(7)* (*see* **Note 1**).

2.2. Agrobacterium tumefaciens *Strains and Binary Vectors*

1. *Agrobacterium* strain EHA101 *(9)*: A derivative of the nopaline type C58 strain and contains the supervirulent disarmed Ti plasmid Bo542 (*see* **Note 2**).
2. Binary vector pFAJ3000 *(10)*: A derivative of the octopine type pGSC1700 vector *(11)*, contains an aminoglycoside adenyltransferase marker gene (*aadA*), which confers bacterial resistance to spectinomycin/streptomycin and a T-DNA with P35S-*uidA*^{INT}-T35S (35S RNA promoter of the cauliflower mosaic virus, intron-

interrupted *uidA* gene, 35S RNA terminator) and Pnos-*npt*II-Tocs (nopaline synthase gene promoter, neomycin phosphotransferase gene, octopine synthase gene terminator) as chimeric plant screenable and selectable marker genes, respectively.

2.3. Culture of Agrobacterium tumefaciens

1. Spectinomycin: Stock solution in Milli-Q water at 100 mg/mL. Filter sterilize and store in 1 mL aliquots at –20°C.
2. Streptomycin: Stock solution in Milli-Q water at 300 mg/mL. Filter sterilize and store in 1 mL aliquots at –20°C.
3. Acetosyringone: Stock solution in dimethylsulfoxide at 500 m*M* (e.g., 98 mg in 1 mL); there is no need to sterilize but solution should always be prepared freshly.
4. Zeatin: Stock solution at 1 m*M*. Dissolve 10.96 mg chemical in a few drops of 1 *M* sodium hydroxide, dilute to 50 mL with Milli-Q water, and store at 4°C.
5. 2,4-Dichlorophenoxyacetic acid (2,4-D): Stock solution at 5 m*M*. Dissolve 55.25 mg 2,4-D in a few drops of 1 *M* sodium hydroxide, dilute to 50 mL with Milli-Q water, and store at 4°C.
6. YM solid medium: 0.1 g/L NaCl, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L $K_2HPO_4 \cdot 3H_2O$, 0.4 g/L yeast extract, 10 g/L mannitol, and 13 g/L bactoagar, pH 7.0. Supplement with the appropriate antibiotics (100 mg/L spectinomycin and 300 mg/L streptomycin) after cooling the autoclaved medium to 55°C. Dispense 25 mL in sterile 9-cm diameter plastic Petri dishes.
7. YEP liquid medium: 5 g/L NaCl, 10 g/L peptone, and 10 g/L yeast extract, pH 7.5. Supplement with the appropriate antibiotics (100 mg/L spectinomycin and 300 mg/L streptomycin) after cooling the autoclaved medium to 55°C. Dispense 10 mL or 20 mL into sterile 50- or 100-mL Erlenmeyer flasks depending on the scale of the experiment.
8. ZZ liquid medium: Half-strength Murashige and Skoog (MS) basal medium supplemented with 2.0 mg/L glycine, 10 mg/L ascorbic acid, 0.4 mg/L thiamine·HCl, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine·HCl, 1 μ*M* zeatin, 5 μ*M* 2,4-D, and 30 g/L sucrose, pH 5.6. Add 200 μ*M* acetosyringone before use, and filter sterilize.
9. Sterile 24-well titer plates to infect embryogenic suspension cells.

2.4. Culture Media for Cocultivation, Selection, and Regeneration of Transformants

1. Geneticin (G-418): Stock solution in Milli-Q water at 50 mg/mL. Filter sterilize and store in 1 mL aliquots at –20°C. Light sensitive, therefore geneticin-containing media should be stored and incubated in the dark.
2. Timentin (a 15:1 mixture of ticarcillin and clavulanic acid): Stock solution in Milli-Q water at 200 mg/mL. Filter sterilize and store in 1 mL aliquots at –20°C.
3. N^6-benzylaminopurine (BAP): stock solution at 1 m*M*. Dissolve 22.52 mg BAP in a few drops of 1 *M* sodium hydroxide, dilute to 100 mL with Milli-Q water and store at 4°C.
4. Indole-3-acetic acid (IAA): Stock solution at 1 m*M*. Dissolve 8.76 mg IAA in a few drops of 1 *M* sodium hydroxide, dilute to 50 mL with Milli-Q water and store at 4°C.

5. Semisolid ZZ medium: Same as ZZ medium (*see* **Subheading 2.3.8.**) except add 2.5 g/L Gelrite as a solidifying agent. Supplement with 200 μ*M* acetosyringone after cooling the autoclaved medium to 55°C, and dispense 10 mL into sterile 5-cm diameter plastic Petri dishes.
6. Semisolid ZZ medium for selection: Same as semisolid ZZ medium except adjust to pH 5.8. Also include the appropriate selective agent (50 mg/L geneticin) (*see* **Note 3**) and 200 mg/L timentin (*see* **Note 4**) after cooling the autoclaved medium to 55°C, and dispense 10 mL into sterile 5-cm diameter plastic Petri dishes.
7. Semisolid RD1 medium: Half-strength MS basal medium plus 2.0 mg/L glycine, 10 mg/L ascorbic acid, 100 mg/L *myo*-inositol, 0.4 mg/L thiamine·HCl, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine·HCl, 30 g/L sucrose, and 2.5 g/L Gelrite, pH 5.8. Supplement with the appropriate selective agent (50 mg/L geneticin) (*see* **Note 3**) and 200 mg/L timentin (*see* **Note 4**) after cooling the autoclaved medium to 55°C; dispense 1 mL into sterile 24-well titer plates.
8. Semisolid RD2 medium: Half-strength MS basal medium plus 2.0 mg/L glycine, 10 mg/L ascorbic acid, 0.4 mg/L thiamine·HCl, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine·HCl, 10 μ*M* BAP, 30 g/L sucrose, and 2.5 g/L Gelrite, pH 5.8. Autoclave, cool down to 55°C, and dispense 10 mL into sterile 5-cm diameter plastic Petri dishes.
9. Semisolid Reg medium: Full-strength MS basal medium plus 2.0 mg/L glycine, 10 mg/L ascorbic acid, 0.4 mg/L thiamine·HCl, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine·HCl, 1 μ*M* IAA, 1 μ*M* BAP, 30 g/L sucrose, and 2.5 g/L Gelrite, pH 5.8. Autoclave, cool down to 55°C, and dispense 20 mL in sterile 50-mL glass test tubes.
10. Semisolid Prol medium: Full-strength MS basal medium plus 2.0 mg/L glycine, 10 mg/L ascorbic acid, 0.4 mg/L thiamine·HCl, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine·HCl, 1 μ*M* IAA, 10 μ*M* BAP, 30 g/L sucrose, and 2.5 g/L Gelrite, pH 5.8. Autoclave, cool down to 55°C, and dispense 20 mL in sterile 50-mL glass test tubes.
11. Sterile end-cut 1-mL (blue) pipet tips to transfer suspension cells.
12. Four-cm^2 pieces of sterile 50-μm polyester mesh to spread infected cells.
13. Sterile 9-cm diameter filter paper disks to remove excess agrobacteria.
14. Parafilm to seal Petri dishes, 24-well plate, and glass test tubes.
15. Soil mix (type 7, Dockx, Mechelen, Belgium): 40% (w/w) White peat moss, 30% (w/w) black peat moss, 20% (w/w) turf fiber, 10% (w/w) Baltic turf, 80 L/m^3 sand, and 1 kg/m^3 PG mix 14-16-18, pH 5.0-5.5.
16. Fertilizer (granulated, Compo, Deinze, Belgium): 15% (w/w) nitrogen (8.5% nitrate, 6.5% ammonium), 20% (w/w) anhydrous phosphorous (P_2O_5), and 26% (w/w) potassium (K_2O).

3. Methods

3.1. Maintenance of Embryogenic Suspension Culture

1. Maintain established embryogenic cell suspension cultures by subculturing every 2 wk in 70 to 90 mL of liquid ZZ medium shaken at 80 rpm in 250-mL Erlenmeyer flasks and incubated at 25°C under a 12-h photoperiod of 1000 lux.

2. Five to seven days after subculture, adjust the cell suspensions to 33% settled cell volume (*see* **Note 5**).

3.2. Growth and Induction of Agrobacterium Cultures

3.2.1. To Grow and Isolate Single Bacterial Colonies From a Glycerol Stock

1. Using a pipet tip, scratch a small amount from the surface of the frozen bacterial stock (*see* **Note 6**) and drop onto the surface of the selective solid YM plate. Place the frozen bacterial stock immediately back into the freezer to avoid extensive thawing.
2. Using a flamed and cooled wire loop, streak cells across the selective solid YM plate to spread bacteria.
3. Incubate the inoculated selective YM plate upside-down for 48 h at 28°C until single colonies are developed.

3.2.2. To Grow up Agrobacterium in Liquid Medium

1. Pick up 3 to 4 isolated single colonies from the plate using sterile toothpicks or pipet tips.
2. Inoculate one colony per 50- or 100-mL Erlenmeyer flasks containing selective liquid YEP medium containing 100 mg/L spectinomycin and 300 mg/L streptomycin.
3. Incubate cultures in a shaker-incubator for 24 to 30 h at 28°C and 210 rpm to reach an OD_{600} of approximately 1.2 units.

3.2.3. To Induce Agrobacterium Virulence

1. Centrifuge liquid cultures at 3430*g* for 10 to 15 min.
2. Discard supernatant and gently resuspend the pellet in sterile liquid ZZ medium supplemented with acetosyringone. Measure the OD_{600} and adjust with the above medium to 0.4 units (*see* **Note 7**).

3.3. Preparation, Infection, and Co-cultivation of Embryogenic Cell Suspensions

1. Carefully transfer individual samples of 200 µL embryogenic cells adjusted to 33% settled cell volume (approx 50 mg fresh weight of cells) to the wells of a 24-well titer plate using end-cut 1-mL pipet tips (*see* **Note 8**).
2. Carefully add 1 mL of the induced-diluted *Agrobacterium* culture to the wells of the 24-well titer plate containing embryogenic cells to be transformed. Add 1 mL of ZZ medium for untransformed controls. Incubate the plates for 6 h at 25°C and 25 rpm in the dark.
3. Pipet the bacteria-plant cell mixture with end-cut 1-mL pipet tips and gently transfer to spread it evenly over a 50-µm sterile polyester mesh placed on top of a few sterile filter paper disks to remove excess *Agrobacterium* and liquid ZZ medium (*see* **Note 9**).

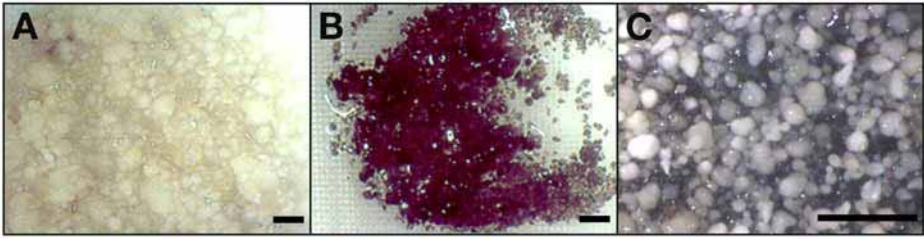

Fig. 1. Selection of transformed banana cell colonies. Control plates of untransformed banana cell cultures **(A,B)** and a plate of banana embryogenic cells transformed with *Agrobacterium* EHA101 (pFAJ3000) **(C)**, cultured in the absence **(A)** and presence **(B,C)** of geneticin at 50 mg/L. Notice the efficiency of the selection **(B)** and the high frequency of induced **(A)** or selected **(C)** embryos. Bar = 2 mm.

4. Individually transfer polyester meshes carrying infected cell samples to 5-cm diameter plastic Petri dishes containing semisolid ZZ medium, pH 5.6. Supplement with acetosyringone (*see* **Note 10**).
5. Incubate cocultivation plates for 6 to 7 d at 21°C in the dark (*see* **Note 11**).

3.4. Selection and Regeneration of Transformants With Stable Expression of the Selectable Marker Gene

1. Transfer the polyester meshes carrying cocultivated samples to sterile 5-cm diameter plastic Petri dishes containing semisolid ZZ medium supplemented with 50 mg/L geneticin and 200 mg/L timentin to select transformed plant cells and inhibit *Agrobacterium* growth, respectively.
2. Incubate selection plates for 2–3 mo at 25 ± 2°C in the dark with subculture to fresh selective plates every 2 wk (*see* **Note 12** and **Fig. 1**).
3. Pick up actively growing cell colonies using sterile forceps, transfer them individually to 24-well titer plates containing semisolid RD1 medium, and label them for subsequent regeneration (*see* **Note 13**).
4. Incubate the 24-well plates for 4 wk at 25 ± 2°C in the dark without subculture.
5. To induce shoots from mature embryos, individually transfer the cultures to sterile 5-cm diameter plastic Petri dishes containing semisolid RD2 medium (*see* **Note 14**).
6. Incubate the cultures for 4 to 6 wk at 25 ± 2°C in the dark without subculture until small shoots appear (*see* **Note 15**).
7. To induce roots and regenerate full-grown in vitro plantlets, transfer differentiated shoots individually to sterile 50-mL glass test tubes containing semisolid Reg medium, and incubate at 25 ± 2°C under a 16-h photoperiod at 1000 to 2000 lux (*see* **Note 16**).
8. Subculture rooted plantlets regularly (every 4–6 wk) to fresh Reg medium to keep transformants healthy.

3.5. Transfer to Soil

1. Carefully remove well-rooted plantlets from the test tubes and gently wash away the semisolid medium with lukewarm tap water.
2. Transfer the plants to soil mix in 12-cm diameter pots individually or to plastic boxes (70 × 40 × 25 cm L × W × H) in groups of 6. Keep the plants under a plastic bag or plastic frame in the greenhouse (26/18°C d/night temperature, 12-h photoperiod at 200-700 lux) for 1 to 2 wk.
3. Gradually increase contact with outside air by raising the plastic cover from the plantlets during a period of 1 wk (see **Note 17**).
4. Water the established plants once a day with tap water and fertilize them with 30 g granulate once in 2 wk. Transgenic lines are maintained as plants, mainly under in vitro conditions.

4. Notes

1. Other cultivars successfully used so far with this protocol to generate transgenic plants are the dessert bananas Grande Naine and Williams (both Cavendish); the plantains Orishele, and Obino l Ewai; the cooking banana Cacambou. The establishment of embryogenic cell suspensions may take 1 to 2 yr *(7)*. The senior author should be contacted for access to embryogenic cell cultures.
2. We have used with success EHA101, EHA105, AGL0, AGL1, and LBA4404 in combination with close to 40 T-DNA constructs based on different vector backbones and mainly octopine or nopaline type borders.
3. Banana cells are naturally resistant to kanamycin (up to 1 g/L, L. Sági, unpublished), therefore geneticin should be used. Geneticin is light sensitive, so store and incubate selective ZZ medium in the dark.
4. At a concentration of 200 mg/L, timentin is effective in eradicating several *Agrobacterium* strains (e.g., LBA4404, EHA101, EHA105, AGL0, AGL1) without evident damage to plant cells.
5. An aliquot of the cell suspension is transferred with a wide-mouth pipet to a sterile graduated cylinder (or any graduated tubes) and allowed to sediment for 5 min. Then, the volume of the culture medium is adjusted so that settled cells make up 33% of the total volume.
6. Bacterial stocks are maintained in 20% (v/v) glycerol-containing growing medium with appropriate antibiotics and stored at –80°C.
7. More dense bacterial cultures may cause overgrowth during cocultivation.
8. Include additional control samples for the transformation and selection process as well as to test transient reporter gene expression. First, culture samples for controlling selection: Place untransformed samples on antibiotic-free semisolid ZZ medium as well as on a medium supplemented with both geneticin and timentin. A transformation control with an empty vector may also be included at the beginning of a new series of experiments or when field trials are planned with the transgenic material. Finally, include several negative control samples, which will not be infected with *Agrobacterium* and are cultured on nonselective media to assess the

regeneration capacity of suspension cells and to provide untransformed control plants for later analyses.

9. This has to be done in two steps to completely collect the mixture from the wells. Do the transfer very gently to avoid cell damage.
10. Cocultivation plates can be prepared the day before the experiment and stored upside-down at 4°C.
11. Cell samples should look uncontaminated at the end of the cocultivation period, without visible overgrowth of bacteria. If cell browning occurs decrease cocultivation time by 1 or 2 d.
12. At the end of the selection period, individual transformation events will be actively growing cell colonies and embryos randomly distributed among dead untransformed plant cells (*see* **Fig. 1**).
13. Alternatively, independent colonies can be transferred to sterile 9-cm diameter plastic Petri dishes containing 25 mL of semisolid RD1 medium, with 9 to 12 well-separated colonies per plate.
14. Alternatively, independent cultures can be transferred individually to sterile 50-mL glass test tubes containing 20 mL of semisolid RD2 medium.
15. At this phase, PCR analysis can be routinely performed, although cell colonies obtained after selection (*see* **Subheading 3.4.3.**) are also suitable. Greenhouse-grown plants are used for Southern analysis. For both methods, total DNA is isolated by the DNeasy plant kits (Qiagen).
16. Shoots can be conveniently proliferated at this stage by transferring to sterile 50-mL glass test tubes containing semisolid Prol medium and incubating for 4 to 6 wk at 25°C under a 16-h photoperiod without subculture.
17. In vitro banana plantlets can be easily acclimatized; a typical survival rate is well above 90% with the described simple procedure.

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17

Citrus

Vladimir Orbović and Jude W. Grosser

Summary

Since the initial reports on production of transgenic Citrus via *Agrobacterium*-mediated transformation, significant progress has been made, and many steps of this procedure using the juvenile tissue explants have been improved. Abundant availability of starting material and relative simplicity make this procedure an attractive choice for many researchers despite transformation efficiency that is in the low range of about 1%. Variety of available *Agrobacterium* strains and reporter/selection genes further facilitate the work by allowing careful planning of experiments in which many steps can be adjusted towards a particular Citrus cultivar. With the use of this procedure, genetically transformed grapefruit, oranges, lime, and rootstock cultivars are routinely produced while some difficulties persist in efforts to transform lemon and especially mandarin cultivars.

Key Words: *Agrobacterium tumefaciens*; genetic transformation; citrus; orange; Carrizo citrange.

1. Introduction

Genetic transformation has become an important approach in programs involved in improvement of Citrus cultivars by enabling researchers to introduce specific genes into already existing superior cultivars while maintaining cultivar integrity. Although efforts have been made in obtaining transgenic Citrus through protoplast transformation by direct DNA introduction (*1,2*) and by employment of *Agrobacterium rhizogenes* (*3*), the most frequently used method of genetic transformation of Citrus involves utilization of *Agrobacterium tumefaciens* (*4–10*).

Accomplishments in the field of genetic transformation of different members of Citrus family have recently been reviewed by Peña and his colleagues (*11*). The same group also most recently described early events in the process of *Agrobacterium*-mediated transformation and pointed towards the cells in cambial region as crucial for successful integration of foreign DNA and generation

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of transgenic plants *(10)*. According to the theory of Peña, cambial cells on the cut surfaces of the explants under specific culture conditions give rise to callus cells that are the major target for *Agrobacterium* action. Furthermore, the efficiency of transformation was linked to the percentage of callus cells that were in S-phase of cell cycle during co-cultivation with *Agrobacterium.* From earlier work, it was determined that *Agrobacterium*-mediated transformation requires cells present in S-phase *(12)*.

Currently, both the juvenile *(4–8)* and mature *(9,10)* tissue of citrus plants can be used as a source of starting material for transformation. The advantage of using mature tissue is production of transgenic plants that overcome the juvenile period, and as a consequence, they flower and bear fruit in less than 15 mo *(9)*. The major disadvantage of this method is difficulty in obtaining ample amounts of sterile starting material for experiments from soil grown plants. The advantages when working with the juvenile tissue are: (1) availability of starting material for transformation obtained under sterile conditions and (2) mechanical features of juvenile tissue that allow easier manipulation. One disadvantage of the method that uses juvenile tissue is the time of about 5 yr to obtain mature, fruit bearing plants. For us, the advantages of using the juvenile tissue outweighed the disadvantages, and we decided to employ this method of transformation.

In our facility, the process of transformation starts with cloning the gene of interest into a binary vector of choice. Upon completion of cloning, a vector carrying the gene of interest, a desired reporter gene, and a gene for antibiotic resistance is mobilized into the appropriate *Agrobacterium* strain. The newly obtained strain of *Agrobacterium* is used to prepare a fresh culture on the day of the experiment. Dark-grown seedlings of Citrus are cut into segments that are approx 15 mm in length. These explants are incubated in suspension of *Agrobacterium* for a short time, blot-dried, and put on nonselective medium supplemented with hormones to facilitate shoot induction. Two days later, explants are transferred to medium containing appropriate antibiotics and shoot inducing hormones. Explants are then left in an incubator for 5 wk. Following this period, the shoots that appear on the explants are harvested and examined for the presence of the reporter gene. Those shoots carrying the reporter gene in their tissue are eventually either grafted onto the rootstock seedlings or induced to create their own roots via culture on rooting media. Rooted and grafted seedlings are moved to soil and grown to a size where their leaves can be used for DNA isolation. Isolated DNA is used for polymerase chain reaction (PCR) to confirm the presence of the target gene in the tissue of putatively transformed plants. Plants that carry the target gene are moved to a greenhouse and grown to maturity.

For a better explanation of the success rate for transformation, we decided to use certain parameters corresponding to steps in the data collection process.

Table 1
Percentage of GUS Positive Citrus Shoots, and Efficiency of Transformation for "Valencia" Orange (Gene of Interest from pTLAB19) and Carizzo Citrange (Gene of Interest from pIB6)

Cultivar	Number of explants	Number of shoots	SMI	% of GUS(+) shoots	Transformation efficiency = %
'VA'	2239	2414	1.08	10.44	1.25
C	806	898	1.11	7.48	1.12

The Shoot Morphogenesis Index (SMI) is a ratio of the number of shoots per number of explants on which shoots were found. For example, if 132 shoots are formed on 100 explants, the SMI is then 1.32. Usually, only 40 to 50% of the shoots that are found on explants are large enough to allow manipulation and use in subsequent tests. Percentage of β-glucoronidase (GUS) positive shoots is calculated as the ratio of those that stained blue per total tested. Finally, the overall transformation efficiency is expressed as a ratio of the number of soil-adapted transgenic plants for which it was confirmed by polymerase chain reaction (PCR) to carry the gene of interest per initial number of the explants used. We know that presence of amplification product in PCR reaction using genomic DNA of soil-adapted *gus* 'positive' plants is not the most definitive proof that those plants are transgenic for the desired gene. However, some of our clients communicated to us that on a small sample of plants that we produced and for which Southern assay was done, 100% of them had gene of interest incorporated into their genome. Also, the *gus* gene present on the binary vector that we used has an intron that precludes its expression in *Agrobacterium,* thereby increasing the chance that the *gus* gene is expressed only in plant cells.

Two examples of transformation achieved in our facility are presented in **Table 1**. The first set of data comes from the transformation of sweet orange (*Citrus sinensis* L. Osbeck 'Valencia') with a gene of interest. The SMI for explants of 'Valencia' orange was 1.08 and the percentage of GUS positive shoots 10.44%. Transformation efficiency was calculated to be 1.25%. The second set of data is the outcome of experiments with explants of Carrizo citrange cultivar [*Citrus sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf.], a popular Citrus rootstock. The calculated SMI for Carrizo explants was 1.11, the percentage of GUS positive shoots 7.48%, and efficiency of transformation was 1.12%. On average, the transformation efficiency achieved in our facility is about 0.9%.

Selection criteria employed by this method can lead to a decrease in recorded efficiency of transformation because of the phenomenon of silencing of transgenes *(5)*, (*see* **Note 1**). Another type of event that may affect transformation

efficiency is breakage of T-DNA during the transfer into the target cell, a problem that has been encountered by other researchers *(7)*, (*see* **Note 2**).

2. Materials

2.1. Plant Material

1. Seeds of sweet orange (*Citrus sinensis* L. Osbeck 'Valencia') and Carrizo citrange [*Citrus sinensis* (L.) Osbeck × *Poncirus trifoliata* (L.) Raf.] are obtained by extraction from harvested fruit.
2. Stems of etiolated plants obtained from seeds are cut into segments that are 15 mm in length and represent material that will be used for incubation with *Agrobacterium*.

2.2. Agrobacterium *Strains and Plasmids*

1. Three strains of *Agrobacterium tumefaciens* are used in our facility for this method of transformation: EHA101 *(13)*, EHA105 *(14)*, and AGL-1 *(15)*.
2. We used two pCAMBIA2301 based constructs (pTLAB19 and pIB6) carrying two different genes of interest cloned into the Pst I site of multiple cloning site. Binary vector pCAMBIA2301 *(16)* is a construct carrying *gus* and *npt*II genes on T-DNA and a kanamycin resistance gene for bacterial selection (*see* **Note 3**).

2.3. Media and Stock Solutions

1. 1X Murashige and Skoog (MS) *(17)* basal medium (Sigma, St. Louis, MO; cat. no. M5519).
2. 1X MS basal salt mixture (Sigma, St. Louis, MO; cat. no. M5524).
3. 100X Murashige and Tucker (MT) *(18)* vitamin stock: 10 g/L of myo-inositol, 1 g/L of thiamine-HCl, 1 g/L of pyridoxine-HCl, 0.5 g/L of nicotinic acid, and 0.2 g/L of glycine. Dissolve in water and store at 4°C.
4. Seed germination medium: 1X MS basal medium with 25 g/L of sucrose and 8 g/L of agar, pH 5.8. Glass tubes (25 × 150 mm) with this medium are stored at room temperature and used within 2- to 3 d after preparation.
5. Stock solutions of growth substances: 1 mg/mL of 6-benzylaminopurine (BA), 1 mg/mL of α-Naphthalene-acetic acid (NAA) and 1 mg/mL of 2,4-dichlorophenoxyacetic acid (2,4-D). Prepare by dissolving the powder in couple of drops of 5 *M* NaOH and bring to final volume with water. Store at 4°C.
6. 9.8 mg/mL acetosyringone stock solution:. Dissolve 196 mg of acetosyringone powder in 20 mL of 50% ethanol. Store at –20°C.
7. CCM-co-cultivation medium *(6)*: 1X MS basal medium with 30 g/L of sucrose plus 3 mg/L of BA (3 mL of BA stock solution), 0.1 mg/L of NAA (0.1 mL of NAA stock solution), 0.5 mg/L of 2,4-D (0.5 mL of 2,4-D stock solution), 19.6 mg/L of acetosyringone (2 mL of acetosyringone stock solution), and 8 g/L of agar, pH 6. Petri-dishes (15 ×100 mm) with this medium are kept at room temperature for a maximum of 3 wk (*see* **Note 4**).
8. RM-Regeneration medium *(6)*: 1X MS basal medium with 30 g/L of sucrose plus 3 mg/L of BA (3 mL of BA stock solution), 0.5 mg/L of NAA (0.5 mL of NAA

stock solution), 333 mg/L of cefotaxime (1.33 mL of cefotaxime stock solution), 8 g/L of agar, and a choice of other appropriate antibiotics, pH 6. For the experiments described herein, we supplemented RM with 70 mg/L of kanamycin (1.4 mL of kanamycin stock solution). Petri-dishes (20 × 100 mm) with this medium are stored at room temperature for a maximum of 7 d.

9. GM-Growth medium: 1X MS basal medium with 25 g/L of sucrose plus 50 mg/L of cefotaxime (0.2 mL of cefotaxime stock solution), 20 mg/L of kanamycin (0.4 mL of kanamycin stock solution), and 8 g/L of agar, pH 5.8. Petri dishes (20 × 100 mm) with this medium are kept at room temperature for up to 4 wk (*see* **Note 5**).
10. Grafting medium: 1X MS basal salt mixture, 100X MT vitamins, 70 g/L sucrose, and 8 g/L of agar, pH 5.8. Glass tubes (25 × 150 mm) with this medium are kept at room temperature for up to 4 wk.
11. Rooting medium ***(19)***: 1X MS basal medium with 25 g/L of sucrose supplemented with 0.5 mg/L of NAA (0.5 mL of NAA stock solution), 0.25 g/L of activated charcoal, and 8 g/L of agar, pH 5.8. Magenta boxes with rooting medium are kept at room temperature for up to 4 wk.
12. YEP-*Agrobacterium* medium ***(20)***: 10 g/L of bacteriological peptone, 10 g/L of yeast extract, and 5 g/L of NaCl, pH 7.0. For solid medium add 15 g/L of agar. For the *Agrobacterium* strains used in experiments, YEP is supplemented with 50 mg/L of rifampicin (1 mL of rifampicin stock solution) and 50 mg/L of kanamycin (1 mL of kanamycin stock solution). Plates (15 × 100 mm) with this medium are kept at 4°C for up to 4 wk.
13. GUS assay solution: To prepare 10 mL, add 5 mL of 0.2 *M* Na_2HPO_4 (14.2 g of dibasic Na_2HPO_4 dissolved in 500 mL of water), 0.2 mL of 0.5 *M* Na_2EDTA (186.1 g of ethylenediamine-tetraacetic acid dissolved in 500 mL of water), 10 mg of X-gluc (cyclohexylammonium salt), and 4.8 mL of water, pH 7.0. Keep at 4°C for up to 7 d.
14. GUS assay fixing solution: Mix 3 parts of 95% ethanol and one part of glacial acetic acid. Keep at 4°C for up to 7 d.
15. Stock solutions of antibiotics: Cefotaxime stock of 250 mg/mL made by dissolving cefotaxime in water; filter sterilized and kept at –20°C. Rifampicin (Rif) stock of 50 mg/mL made by dissolving rifampicin in DMSO; kept at –20°C. Kanamycin (Kan) stock of 50 mg/mL made by dissolving kanamycin sulfate in water; filter sterilized and kept at –20°C.
16. Soil: Metro-Mix 500 growing medium (Scotts). All media are autoclaved at 116°C and 1.5 bar for 20 min. Growth substances are added to the medium before autoclaving. Acetosyringone and antibiotics are added to the medium after it was autoclaved and cooled down to 55°C.

3. Methods

3.1. Preparation of Plant Material

1. Seeds are peeled with fine jeweler forceps and surface-sterilized by hand-shaking for 15 min in 20% solution of commercial bleach. These seeds are then rinsed with sterile water three times, 20 min for each rinse.

2. Place two seeds per glass tube containing seed germination medium. Cap the tubes containing seeds, seal them with Nescofilm, and incubate them in the dark at room temperature (25 ± 4°C) for 5 wk. During this period, seedlings will germinate from the seeds and grow to be about 10- to 12-cm long.
3. On the day of the experiment, take seedlings out of the tube using sterile forceps and place them on sterilized paper plates.
4. Using a sterile surgical blade mounted on a scalpel handle remove root, a portion of the stem carrying cotyledons, and apical hook (*see* **Fig. 1**).
5. Cut the remaining seedling into pieces (about 15-mm long) so that both ends of each explant are slanted (*see* **Note 6**).
6. Place explants (no more than 250) in a Petri dish (20 × 100 mm) containing liquid CCM (about 30 mL) and leave them there (usually 1–3 h) until the time of transfer to the *Agrobacterium* suspension.

3.2. Preparation of Agrobacterium Cultures

1. Two days before the experiment (*see* **Note 7**), start liquid cultures of *Agrobacterium* by inoculating 50 mL of YEP+Rif+Kan medium with one colony. These cultures are placed in an incubator that maintains the temperature at 28°C and a shaking speed of 220 rpm.
2. One day before the experiment, examine the culture and if it appears dense and opaque (optical density-OD_{600} higher than 1), replenish the medium and antibiotics in the bacterial culture. Take 2 mL of growing culture using a sterile pipett and discard the rest. Use the culture from the pipett as an inoculum for the new culture with fresh YEP and antibiotics. If the culture is growing slowly and is translucent, leave it in the incubator without changing the medium or incubation conditions.
3. On the day of the transformation experiment, examine *Agrobacterium* culture. It should be growing vigorously at this time. Replenish the medium and antibiotics in the culture to obtain actively growing bacteria for maximum effect at the desired time. Allow this culture to grow for additional 4 to 5 h.
4. Harvest 35 ml of *Agrobacterium* culture by centrifuging it at 3000*g* for 10 min then resuspend the pelleted bacteria in 35 mL of liquid CCM medium.
5. The optical density (OD_{600}) of resuspended culture is measured and adjusted to the desired level with additional amounts of CCM. Choice of Citrus cultivar affects the optical density of bacterial suspension used in the experiment (*see* **Note 8**). For strains AGL-1 + pTLAB19 (used with 'Valencia' explants) and EHA101 + pIB6 (used with Carrizo explants) we have adjusted OD_{600} to 0.5.

3.3. Co-incubation of Explants With Bacteria

1. Take a group of explants (about 50) out of the plate with CCM and place them on sterilized paper towel to remove most of CCM liquid. This step, which takes about 1 min, is necessary to avoid further dilution of bacterial suspension by each consecutive group of explants carrying some CCM.
2. Transfer the explants to the Petri dish (20 × 100 mm) with 30 mL of *Agrobacterium* suspension where they should be soaked for 1 to 2 min.

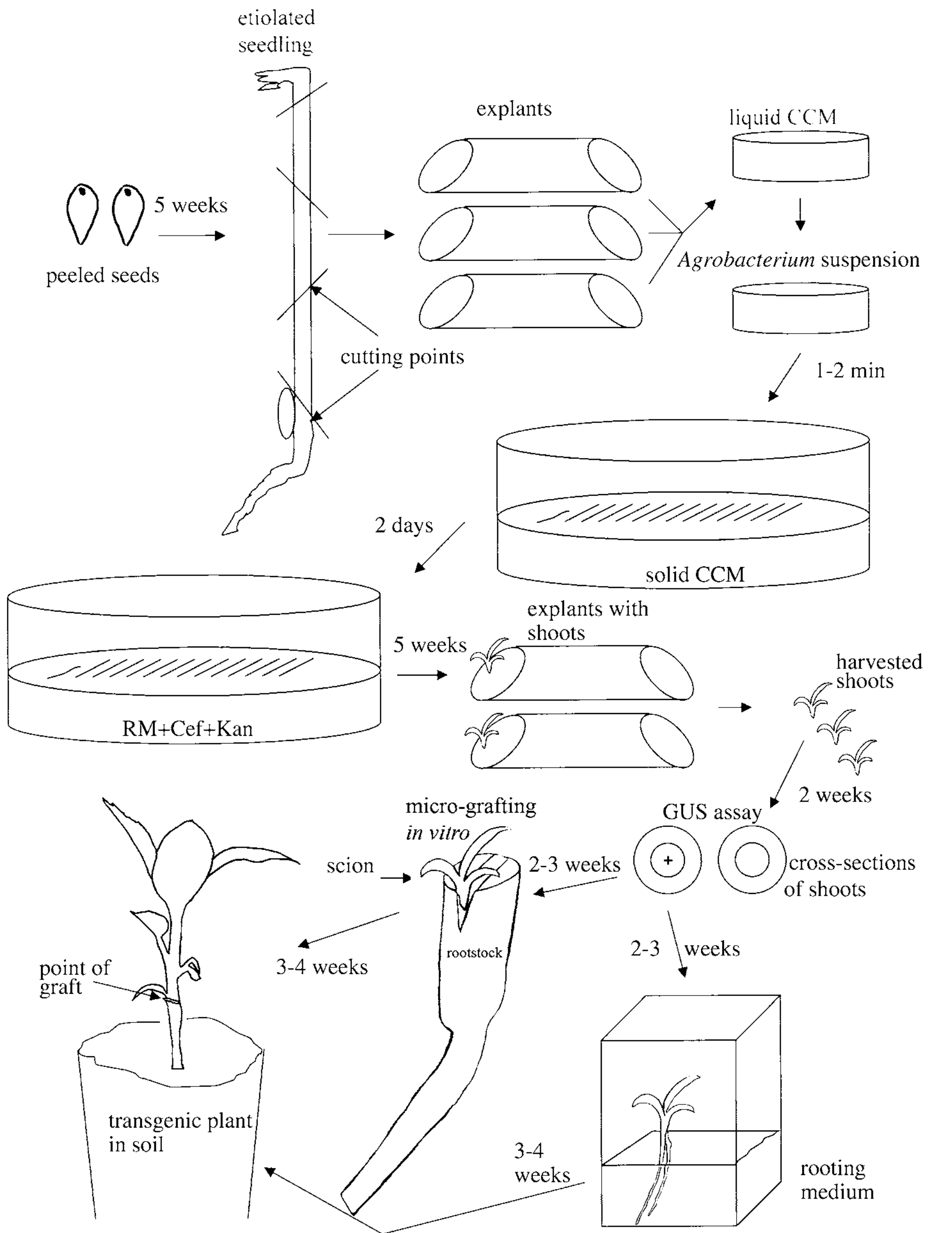

Fig. 1. Flow-chart depicting phases in the process of transformation (not to scale).

3. Following co-incubation with bacteria, place the explants on sterilized paper towels again to remove the excess of bacterial suspension. Because in the next step explants are placed on solid CCM medium that does not contain any antibiotics,

getting rid of excess of bacteria is needed to prevent their overgrowth that may cause loss of the explants.

4. Place 16 infected explants on plate with solid CCM. Seal plates containing the explants with Nescofilm and place them in the incubator for 2 d. Temperature in the incubator is maintained at 26.1°C for 16 h photoperiod (35 µmol/m^2/s) and at 24.5°C for 8 h of dark.
5. Transfer explants from plates with CCM to RM medium, seal plates, and leave them in the incubator for about 5 wk. Incubation conditions same as in **step 4**.

3.4. Testing of Shoots for Presence of Reporter Gene

Thirty to thirty-five days after co-incubation with *Agrobacterium*, inspect the explants for the presence of shoots.

3.4.1. β-glucuronidase as a Reporter Gene

1. To test for β-glucuronidase ***(21)*** as a reporter gene for transformation, harvest the shoots by excising them from the explants and culture them on GM medium (*see* **Note 5**). At this time, make sure that all the tissue that may be leftover from the explant is removed from the shoot.
2. Plates with 96 microassay wells are convenient for this type of test. Aliquot 50 µL of GUS assay solution (*see* **Note 9**) into each well and place the cut pieces of shoots to be tested into those wells. The pieces that are used for assay should come from the bottom of the shoot, represent complete cross section, and be as thin as possible.
3. Seal the plate with Nescofilm and leave it at 37°C for at least 4 h.
4. After this period of time, remove the film and add 50 µL of fixing solution into each well.
5. Observe the samples 1 h later after chlorophyll has been bleached. At that time, GUS staining will be easier to score.
6. Select the shoots corresponding to the cross sections that exhibited blue GUS staining and transfer them to fresh GM medium.

3.4.2. Green Fluorescent Protein as a Reporter Gene

1. If you used Green Fluorescent Protein (GFP) ***(22)*** as a reporter gene for transformation, observe shoots under the "fluorescent" microscope. This is a binocular microscope that has a source of blue light attached to it. When blue light hits molecules of GFP, it excites them to emit green light. As a result of the presence of GFP in transgenic tissue, shoots appear completely green, light pink, or red with green patches of different size. Under the same conditions, wild-type shoots appear red because chlorophyll emits red light after excitation with blue light. The pink color of shoots indicates presence of low levels of GFP in the tissue and the concomitant emission of light by both GFP and chlorophyll. Following selection, excise transgenic shoots from the explants and place them on GM medium.

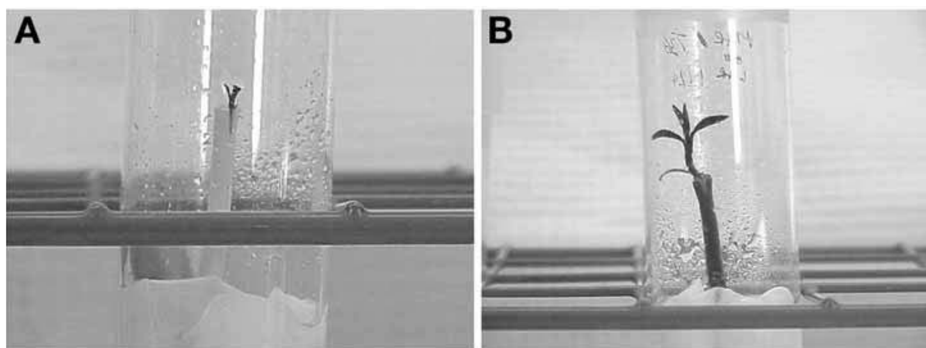

Fig. 2. Photographs of micro-grafted shoot **(A)** Immediately after grafting **(B)** 25 d after grafting.

3.5. Micrografting of Transgenic Shoots In Vitro

This technique, described by L. Peña *(4)*, requires a worker with calm nerves and good hand skills. All of 'Valencia' sweet orange transgenic shoots should be grafted on appropriate rootstock as it is hard to induce roots on these plantlets in vitro.

1. Plants that are used as a rootstock are grown the same way as the plants that are used to obtain explants (see **Subheading 3.1.**), meaning they should be fully etiolated on the day of grafting. For rootstock, select only plants which have at least 5 cm of straight shoot and root when measured from cotyledons (*see* **Note 10**).
2. Pull the plant out of the tube with sterile forceps, make a transverse cut about 2 cm above cotyledons with a sterile surgical blade mounted on scalpel handle, and discard the stem.
3. Cut the root about 4 to 5 cm below cotyledons and remove root hairs if there are any. At this point, everything should be done as quickly as possible to prevent the drying of cut surfaces of plant tissue.
4. Take the putative transgenic shoot and carefully cut it at the bottom so that lowest portion of the stem appears as a letter V.
5. Make a 2- to 3-mm deep longitudinal cut along the center of the cut surface of rootstock with a surgical blade. While the blade is in the rootstock, wiggle it to the left and right so that the slit widens a little and is ready to accept the graft.
6. Insert the wedged part of the shoot into the slit of rootstock, and transfer this plant into the tube with grafting medium (*see* **Figs. 1** and **2**). We micro-graft all transgenic 'Valencia' shoots on Carrizo rootstock (*see* **Fig. 2**). Tubes with grafted plants are left in the incubator for 3 to 4 wk. During this time, scions that have been successfully grafted develop into young plants (*see* **Fig. 2**), and roots of the rootstock increase in size and grow secondary roots and root hairs. Temperature in the incubator where tubes with these plants are kept is maintained at 26.1°C for 16-h photoperiod (35 $\mu mol/m^2/s$) and at 24.5°C for 8-h of dark. Our typical rate of grafting success is about 75%.

7. Grafted 'Valencia' orange plants that have grown in vitro to the size depicted in Fig. 2 should be transferred to soil and grown in the laboratory on the light bench at room temperature (25 ± 4°C) and 24-h photoperiod (55 μmol/m^2/s) for 2 to 3 mo. During this time, plants are watered twice a wk and fed once a wk with a fertilizer solution (1 g/L of 15:30:15 NPK Deep feeding formula-Sam's choice). These plants are used for PCR testing and depending on the outcome either discarded or delivered to clients.

3.6. Root Induction on Transgenic Shoots

1. Root induction in vitro is achieved relatively easy for shoots of Carrizo citrange, and for that reason we do not graft them, we induce root morphogenesis in these plantlets.
2. Following the 2 to 3 wk period of incubation on GM growth medium, transfer putatively transgenic Carrizo shoots to rooting medium. When doing the transfer, make sure to make a fresh cut at the base of the shoot so that exposed tissue gets in contact with the medium into which it is plunged. Once roots grow to be at least 4 to 5 cm in length, transfer the plants to the soil (*see* **Fig. 1**) and treat them the same way as grafted plants (*see* **Subheading 3.5.7.**).

4. Notes

1. In one series of experiments with explants of 'Marsh' grapefruit (data not shown), we selected a group of 14 shoots that did not stain blue in GUS assay but exhibited vigorous growth on selective medium. These shoots were grafted and used as a source of tissue for DNA isolation. When PCR reaction was performed with these samples as sources of template, it was shown that five shoots carried the gene of interest in their tissue. Because of this, transformation efficiency in our experiments may have been higher than indicated by the data presented in **Table 1**.
2. In the series of experiments with 'Valencia' orange explants (**Table 1**), genomic DNA from plants that stained blue in GUS assay was used to perform PCR with primers that would result in amplification of the GUS sequence. The number of plants that carried GUS gene in their tissue was 34. However, only 28 of them also carried the LTP gene.
3. Binary vectors were mobilized into *Agrobacterium* by a freeze-and-thaw method (*23*). Once the binary vector of interest has been mobilized into an appropriate strain, two stocks of newly created strains are made. Glycerol stock is kept at −80°C and working stock is kept at 4°C on plates of YEP medium supplemented with appropriate antibiotics.
4. Liquid CCM is used for temporary incubation of cut explants before they get infected in *Agrobacterium* suspension. Solid CCM is used for incubation of explants following incubation with *Agrobacterium*.
5. GM medium is used for incubation of putatively transgenic shoots already used in GUS assay. Also, GM is used for 2 to 3 wk incubation of smaller shoots harvested from the explants. This allows them to grow to a size of 5 to 6 mm when they are easy to manipulate and be used in GUS assay.
6. There is a report stating that transformation efficiency increases if explants are cut longitudinally as a result of higher surface of cambial tissue being exposed to

action of *Agrobacterium* (*6*). We confirmed these results (data not shown) but also noticed that stems of many Citrus cultivars are not sturdy enough to sustain longitudinal cuts. For that reason, as well as for labor efficiency, we chose not to cut explants longitudinally but made slanted, instead of transversal, cuts on ends of explants, thereby increasing the surface area of internal tissues that came in contact with *Agrobacterium*.

7. Cultures of *Agrobacterium* start to lose their viability when kept on YEP+Rif+Kan plates at 4°C for more than 3 wk. Because of that, it is prudent to start the culture for experiment 2 d earlier to allow bacteria time to attain vigorous growth; a 24-h period may not be enough.
8. Although we never made exact calculations, we have noticed that different cultivars of Citrus go through co-incubation with *Agrobacterium* differently. Carrizo citrange and all grapefruit cultivars stand up well to treatment by EHA101 (the most virulent) strain of *Agrobacterium*. On the other hand, cultivars of sweet orange and lemons do not stand co-incubation with EHA101 well, and some explants (10–20%) turn brown and shrivel within 2 to 3 wk. For transformation of these citrus cultivars, we use either EHA105 or AGL-1 strains. Also, for the latter, more sensitive cultivars, OD_{600} of *Agrobacterium* suspension is usually set to 0.3- to 0.5 when using EHA105 and at 0.5 with AGL-1. EHA101 suspension is used with its OD_{600} set between 0.5 and 0.7.
9. We only make 10 mL of GUS assay solution at the time because it is not stable for a long time at 4°C.
10. Tubes used for growth of grafted plants have special floaters made out of circular, 9-cm diameter filter paper. Liquid medium should be poured into the tubes before the floaters are installed as floaters need to stay dry. These floaters are made as follows: paper is put over the top of the tube—(the center of the paper should overlap the center of the tube opening)—and pushed downwards so it wrinkles and folds around the tube; only a small platform covering the tube opening stays flat. At this time, the paper looks like a makeshift cover for the tube. The next step is to take a sharp object and make a hole (2–3 mm) in the center platform part of the "paper cover" of the tube. Paper is removed from the tube and pushed carefully into the tube so the platform with the hole stays at the top and is a few millimeters below the opening of the tube. A floater prepared like this is ready to receive the rootstock with grafted shoot and to be pushed to the bottom of the tube. Tubes are capped and autoclaved after both medium and the floaters have been put in. Because of this set-up, it is necessary to have the root of the rootstock plant straight so it can easily go through the wrinkled part of the floater. Also, it is beneficial if the stem of the rootstock is straight because the grafted shoot stays in the center of the tube once the floater carrying the rootstock is pushed down the tube and plunged into the medium.

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18

Coffee (*Coffea* sp.)

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Summary

Coffee (*Coffea* sp.) is a perennial plant widely cultivated in many tropical countries. It is a cash crop for millions of small farmers in these areas. As compared with other tree species, coffee has long breeding cycles that make conventional breeding programs time consuming. For that matter, genetic transformation can be an effective technique to introduce a desired trait in an already "elite" variety, or to study a gene function and expression. In this chapter, we describe two *Agrobacterium*-mediated transformation techniques; the first with *A. tumefaciens* to introduce an insect resistance gene and the second with *A. rhizogenes* to study candidate gene expression for nematode resistance in transformed roots.

Key Words: *Agrobacterium tumefaciens*; *Agrobacterium rhizogenes*; insect resistance; nematode resistance; *Bacillus thuringensis*; *Meloidogyne* sp.; functional genomics.

1. Introduction

With more than 7 million tons of green coffee beans produced every year on about 11 million hectares all over the intertropical countries. Coffee is an extremely important agricultural crop. In terms of economic importance on the international markets it is second only to oil and contributes more than 9 billion US dollars.

Traditional breeding is aimed at improving the income of the planters, who are mainly small farmers. As other perennial crops, coffee has a long juvenile period. Conventional breeding can take between 25 and 35 yr. It is a major drawback for coffee improvement. Genetic engineering could shorten this time by allowing the incorporation of known genes into elite genetic backgrounds.

Two major species, *Coffea arabica* (self-pollinated and allotetraploid: $2n = 44$, 68% of the global production) and *Coffea canephora* (self-sterile and diploid: $2n = 22$) are cultivated all over tropical areas. Arabica breeding is traditionally based

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on pure line selection, but recently, an F1 hybrid selection strategy has been developed (*1,2*). Desired traits to be inserted into elite varieties are principally increased yield and beverage quality along with pest and disease resistance. *C. canephora* breeding is more oriented towards improving yield and technological and organoleptic qualities through creation of hybrids between genotypes of different genetic groups (*3*) or selection of improved clones (*4*).

Works on genomics and gene mining have recently been developed. Genetic transformation is also a tremendous tool for studying the function and expression of genes (e.g., genes involved in coffee quality or in resistance to diseases and parasites) or for understanding mechanisms associated with the introgression of foreign germplasm within a species. Mastering introgression, primarily from *C. canephora* to *C. arabica*, poses a major challenge for the next 20 yr.

Reviews on biotechnology techniques applied to coffee have recently been published (*2,5*). Most studies deal with large scale propagation techniques through somatic embryogenesis, somaclonal variation, in vitro preservation of coffee germplasm, genetic transformation, and evaluation and use of genetic resources based on the utilization of molecular markers.

The major pests threatening production are coffee leaf rust, coffee berry disease, leaf miner, and nematodes. The leaf miner *Leucoptera* spp. has an economically important impact in East Africa and Brazil (*6*). Because the caterpillar develops inside coffee leaves, insecticide sprays do not affect it. For a treatment to be efficient, the miner must ingest the insecticide or insecticidal protein. This implies the use of systemic insecticides which are harmful to the environment. A valuable strategy would then be the transgenic approach. The use of *Bacillus thuringiensis* genes to transform plants for protection against insects is currently the most reliable strategy (*7,8*) and preliminary investigations were therefore conducted to determine the susceptibility of *Leucoptera* spp. to *B. thuringiensis* insecticidal proteins and identify candidate genes for the transformation of coffee (*9*). The toxin expressed by the *cry*1Ac gene, widely used to confer resistance to Lepidopterae (*10*), has been demonstrated to be the most effective. Current studies are in progress to select other *B. thuringiensis* strains that may be active against coffee berry borer and white stem borer (*11*), two coleopteran pests of economic importance.

The main cultivated *C. arabica* varieties are dwarfs—which are high yielding and produce good quality coffee—but they prove susceptible to numerous pathogens, particularly endoparasitic root nematodes of the genus *Meloidogyne* (*12*). In many production regions, and more particularly in Brazil and Central America, *Meloidogyne* spp. (root-knot nematodes) is a major agricultural constraint. Attacks have a considerable impact and they can substantially reduce yields, kill trees, and lead farmers to cut-off coffee trees. Nematicide chemicals are expensive, not very efficient, and harmful to both humans and the environ-

ment. It is unanimously accepted that the way to fight root-knot nematodes is to select resistant varieties. Sources of specific resistance to *Meloidogyne* have been identified in diploid species and the gene of resistance to *M. exigua* (*Mex1*) will be shortly isolated ***(13)***. The regeneration of hairy roots after transformation with *A. rhizogenes* has been reported in a large number of species ***(14)***. Hairy roots are used to study mycorrhization, nodulation and nitrogen fixation, and for the production of secondary metabolites under controlled conditions in bioreactors, as well as for studying plant/nematode interaction (***15***). Our team has developed the production of roots transformed by *A. rhizogenes* to validate candidate genes for resistance to root-knot nematodes (e.g., "*Mex1*") by functional complementation, and to study the conditions required for expression of such genes. The ultimate objective is to breed varieties using molecular marker-assisted selection (MAS), into which genes of resistance to the different nematodes have been pyramided.

Genetic transformation of coffee was first performed on cells using protoplast electroporation ***(16)***. Genetic engineering using *Agrobacterium* sp. has also been reported ***(17,18)***. Regeneration of transgenic coffee trees was obtained after transformation of somatic embryos via *Agrobacterium rhizogenes* ***(19–21)***, or via *Agrobacterium tumefaciens* ***(22–24)***. Although somatic embryogenesis is still a tedious process for some coffee species ***(25,26)***, regeneration was easily obtained.

Recent studies have been conducted on pests and diseases resistance genes ***(13,27)***, cup quality improvement genes ***(28)*** and specific promoters ***(29)***. Genetic tools such as linkage mapping ***(30,31)*** and BAC DNA libraries ***(32,33)*** can be useful for new gene searches.

Suitable transformation protocols are of utmost importance, either to evaluate gene functionality or to introduce new genes of interest. Starting with an existing procedure ***(19)***, we developed two different protocols. The first using *A. tumefaciens,* aims at introducing new genes and, although we used specific constructs designed for providing insect resistance, our technique can be used with different types of constructs. We developed a high-yield procedure, which is described here, as well as modifications for use with different coffee genotypes.

Our methodology for coffee genetic transformation involves somatic embryos or immature embryogenic cultures or suspensions. We were able to apply this methodology to 20 different genotypes among the two cultivated coffee species *C. arabica* and *C. canephora*. Field study is an important step in genetic engineering studies and our preliminary results show consistency with laboratory bioassays as well as good agronomic behavior. The results of a 4-yr field trial in French Guyana show that the transformed coffee trees express an efficient resistance to the pest ***(34)***.

The second protocol we describe here has been adapted to study the expression of genes of resistance to root-knot nematodes. It consists of the rapid regeneration of hairy roots after transformation of zygotic embryos by *A. rhizogenes* (*see* **Note 1**). This protocol was developed for coffee based on those published for other species (*35,36*). Our procedure enables rapid and routine generation of hairy roots, as well as their maintenance. Two *Coffea arabica* genotypes were used for these experiments. The Caturra variety, which is widely grown in Latin America, is susceptible to the root-knot nematode *Meloidogyne exigua*. The second variety used, Iapar 59 (a Catimor-type variety), is resistant to numerous diseases and pests. Its resistance to *Meloidogyne exigua* has been demonstrated (*37*).

2. Materials

2.1. Regeneration of Whole Transgenic Plants Using *A. tumefaciens*

2.1.1. Plant Materials

Leaves from greenhouse grown mature plants of *Coffea canephora* and *Coffea arabica*.

2.1.2. Transformation Vectors

1. *Agrobacterium tumefaciens* strains: LBA4404, C58, and EHA105.
2. Vector background: Construct has been integrated in the pBin19 (*38*) and pCambia A1 plasmids.
3. Transgenes:
   a. A screenable marker gene (*see* **Note 2**).
   b. A selectable marker gene (*see* **Note 3**).
   c. A trait gene (*see* **Note 4**).

2.1.3. Culture Media for *Agrobacterium* Strains

1. YEP medium: 10 g/L Bacto-peptone (Difco), 10 g/L yeast extract (Merck), 5 g/L NaCl, pH 7.0.
2. Kanamycin sulfate (Sigma): 50 mg/mL stock solution in water. Sterilize by filtration through a 0.2-µm membrane. Dispense 1 mL aliquots in Eppendorf tubes and store at −20°C (used for the three strains).
3. Streptomycin sulfate (Sigma): 100 mg/mL stock solution in water. Sterilize by filtration and store at −20°C (used for LBA strain).
4. Rifampycin (Sigma): 25 mg/mL stock solution in water, dissolve in 1*N* HCl and bring up the final volume with reverse osmosis (RO) water. Sterilize by filtration and store at −20°C (used for the three strains).
5. Gentamycin (Sigma): 10 mg/mL stock solution in water. Sterilize by filtration and store at −20°C (used for C58 strain).
6. YEP liquid medium containing 50 mg/L kanamycin, 50 mg/L rifampycin, 100 mg/L streptomycin (for LBA strain), or 20 mg/L gentamycin (for C58 strain), pH 7.5.

2.1.4. Stock Solutions

1. 6-Benzylaminopurine (BAP) (Sigma): 10 mg/100 mL stock solution. Prepare by dissolving in 1 mL of 1*N* HCl before making up to 100 mL with RO water. Store at 4°C.
2. 2,4-Dichlorophenoxyacetic acid (2,4-D) (Sigma): 10 mg/100 mL stock solution. Prepare by dissolving in 1 mL of 1 *N* NaOH before making up to 100 mL with RO water. Store at 4°C.
3. 6-(γ,γ-dimethylallylamino)-purine (2-iP, Sigma): 10 mg/100 mL stock solution. Prepare by dissolving in 1 mL of 1 *N* NaOH before making up to 100 mL with RO water. Store at –20°C.
4. Indole-3-butyric acid (IBA) (Sigma): 10 mg/100 mL stock solution. Prepare by dissolving in 1 mL of 1 *N* NaOH before making up to 100 mL with RO water. Store at 4°C.
5. Kinetin (Sigma): 10 mg/100 mL Stock solution. Prepare by dissolving in 1 mL of 1*N* NaOH before making up to 100 mL with RO water. Store at 4°C.
6. Cefotaxime (Claforan, Roussel): 200 mg/mL in water. Sterilize by filtration and store at −20°C.
7. Chlorsulfuron (Kalys, Roubaix, France): 200 µg/mL, use 3*N* KOH for correct dilution, sterilize by filtration and store at −20°C.
8. Acetosyringon (Fluka Chemical cat. no. 38766): 1 m*M* in EtOH, store at –20°C. Shake well before use.

2.1.5. Media for Plant Tissue Culture and Transformation

All media are sterilized by autoclaving for 20 min at 121°C; antibiotics and chlorsulfuron added to autoclaved media after cooled down to 55°C.

1. Yasuda medium ***(25)***: Modified Murashige and Skoog (MS) salts ***(39)*** (Duchefa cat. No. M0221.0001), Gamborg B5 vitamins (***40)***, 10 mg/L thiamine-HCl, 1 mg/L pyridoxine-HCl, 1 mg/L nicotinic acid, 100 mg/L *myo*-inositol), 1.125 mg/L BAP, 30 g/L sucrose, 6 g/L Phytagel™, pH 5.6 (*see* **Note 5**).
2. T1B medium ***(26)***: Half-strength MS salts, 10 mg/L thiamine-HCl, 1 mg/L pyridoxine-HCl, 1 mg/L nicotinic acid, 1 mg/L glycine, 100 mg/L *myo*-inositol, 100 mg/L casein hydrolysate, 400 mg/L malt extract (Sigma), 0.5 mg/L 2,4-D, 1 mg/L IBA, 2 mg/L 2-iP, 30 g/L sucrose, 2 g/L Phytagel™, pH 5.6.
3. T2B medium ***(26)***: Half-strength MS salts, 20 mg/L thiamine-HCl, 20 mg/L glycine, 40 mg/L L-cystein (Sigma), 200 mg/L *myo*-inositol, 60 mg/L adenine sulfate (Sigma), 200 mg/L casein hydrolysate, 800 mg/L malt extract, 1 mg/L 2,4-D, 4 mg/L BAP, 30 g/L sucrose, 2 g/L Phytagel™, pH 5.6.
4. T3B medium ***(26)***: Half-strength MS salts, 5 mg/L thiamine-HCl, 0.5 mg/L pyridoxine-HCl, 0.5 mg/L nicotinic acid, 10 mg/L L-cystein, 50 mg/L *myo*-inositol, 100 mg/L casein hydrolysate, 200 mg/L malt extract, 1 mg/L 2,4-D, 1 mg/L kinetin, 15 g/L sucrose, pH 5.6.
5. Germination medium: Semi-solid MS germination medium with half-strength salts, Morel vitamins ***(41)*** (1 mg/L thiamine-HCl, 1 mg/L pyridoxine-HCl, 1 mg/L nicotinic

acid, 100 mg/L *myo*-inositol, 1 mg/L calcium pantothenate, and 0.01 mg/L biotine), 0.225 mg/L BAP, 15 g/L sucrose, and 3 g/L Phytagel™, pH 5.6.
6. Rooting medium: Identical to germination medium, without BAP.

2.1.6. Other Solutions and Supplies

1. Leaf surface disinfectant solution: 8% HClO (w/v).
2. 50-μm nylon mesh: Ref. 87 mn PA 50/27 (SAATI, Sailly, France).
3. Sand-soil (75:25) mixture: Soil ref. 9 SE A7 (Touchat Technique, Mauguio, France).
4. Commercial rooting solution (0.4% IBA): (Exuberone, Rhodiagri, Lyon, France, cat. no. AMM7400758)

2.2. Hairy Root Regeneration Using A. rhizogenes

2.2.1. Plant Materials

Seeds of *Coffea arabica* var. Caturra and Iapar 59 were obtained at the ICAFE research center of Costa Rica.

2.2.2. Transformation Vectors

The *Agrobacterium rhizogenes* strain A4RS was used for transformation (*see* **Note 6**). This strain derived from the wild strain A4 modified for the resistance to rifampycin and spectinomycin antibiotics *(42)*. Construct has been integrated in the pBin19 plasmid *(38)*. The *uidA* bacterial gene isolated from *Escherichia coli* coding for β-glucuronidase (GUS), was introduced, with an additional intron for specific expression in plants *(43)*. The gene was controlled by the cauliflower mosaic virus (CaMV) 35S promoter and terminator. The A4RS including the pBin19 plasmid with the above construct was called armed A4RS.

2.2.3. Culture Media for Agrobacterium rhizogenes Strains

1. MYA medium: 5 g/L yeast extract (Merck), 0.5 g/L casein hydrolysate (Merck), 8 g/L mannitol, 2 g/L $MgSO_4$, 5 g/L NaCl, and 15 g/L agar, pH 6.6.
2. Kanamycin sulfate (Sigma): 50 mg/mL stock solution in water. Sterilize by filtration through a 0.2-μm membrane. Dispense 1-mL aliquots into Eppendorf tubes and store at −20°C.
3. Rifampycin (Sigma): 25 mg/mL stock solution in water; use a few drops of 1 *N* HCl for correct dilution. Sterilize by filtration and store at −20°C.
4. Spectinomycin (Sigma): 20 mg/L stock solution in water. Sterilize by filtration through a 0.2 μm membrane and store at −20°C.
5. MYA semi-solid medium containing 50 mg/L rifampycin (both for wild and armed A4RS), 500 mg/L spectinomycin (both for wild and armed A4RS), 50 mg/L kanamycin (only for armed A4RS), pH 6.6.

2.2.4. Tissue Culture

Media sterilized by autoclaving for 20 min at 121°C; cefotaxime added to cooled sterile media.

1. GER germination medium: semi-solid MS medium ***(39)*** with full-strength salts (Duchefa Biochemie), regeneration vitamins (10 mg/L L-cystein, 10 mg/L thiamine-HCl, 1 mg/L pyridoxine-HCl, 2 mg/L glycin, 1 mg/L nicotinic acid), 40 g/L sucrose, and 2.5 g/L Phytagel™.
2. Seed surface disinfectant solution: 8% HClO (w/v), Tween-40 (10 droplets).
3. Cefotaxime (Duchefa Biochemie): 200 mg/mL in water. Sterilize by filtration and store at −20°C.

3. Methods

3.1. Prodution of Whole Transgenic Plants Using A. tumefaciens

3.1.1. Leaf Explant Sterilization

1. Cut leaves from mother trees in the greenhouse. Optimal stage is fully expanded leaves, still immature (not as glossy as older leaves).
2. Sterilize the leaves for 20 min with 8% HClO (w/v) and rinse three times with sterile RO water. Cut leaves into small pieces (0.25 cm^2) and culture them (four explants per dish) in 5.5-cm diameter Petri dishes containing 12.5 mL callus initiation media according to the genotypes (*see* **Subheading 3.1.2.**).

3.1.2. Callus Culture Initiation

For *Coffea canephora* genotypes:

1. Culture leaf explants on semi-solid Yasuda (four leaf pieces per plate), at low light (10 μE/m^2/s), 16-h photoperiod, at 27°C. Sub-culture every 5 wk until embryogenic callus appears on the cut edges of the explants (*see* **Fig. 1D**).

For *Coffea arabica* genotypes:

1. Leaf explants are cultured successively on the two media defined for the F_1 hybrid genotypes. First, culture leaf explants on T1B medium in 5.5-cm diameter Petri dishes (4 leaf pieces per plate) for 4 wk in the dark at 27°C.
2. Then transfer the leaf explants to T2B medium in 10-cm diameter Petri dishes (four leaf pieces per plate) and incubate the plates under a low-light condition (10 μE/m^2/s, 16-h/d, 27°C). Sub-culture every 5 wk to fresh T2B medium for 3 to 5 mo until embryogenic callus appears on the cut edges of the explants.

3.1.3. Callus Culture Preparation for Transformation

1. Six wk before transformation, or about 1 mo after callus initiation, sub-culture a low density of embryogenic calli (1 g/dish) onto a 50-μm nylon mesh overlay on

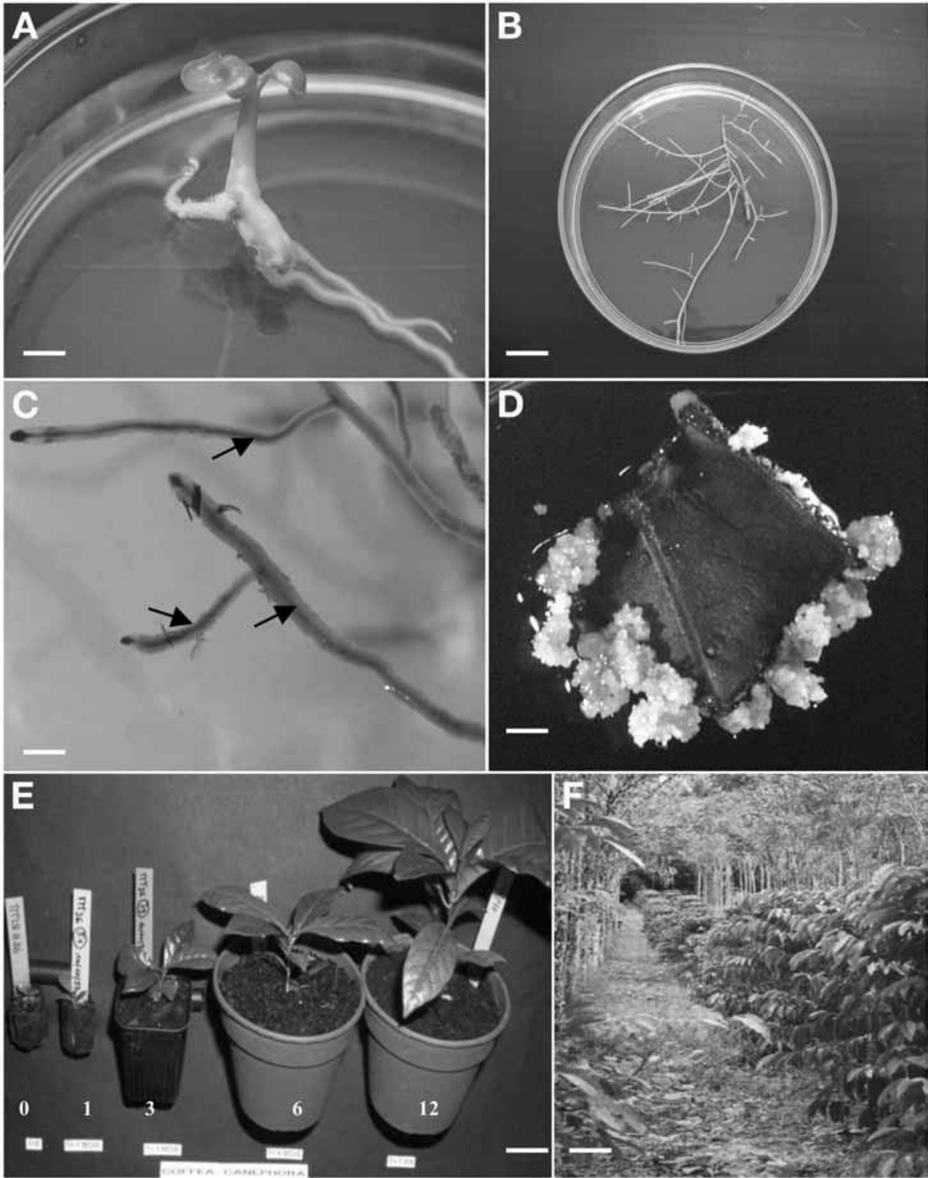

Fig. 1. **(A–C)** Regeneration of coffee transgenic roots (hairy roots) using *Agrobacterium rhizogenes*. **(A)**, Regeneration of a transgenic *C. arabica* root at the wounded and infected site (hypocotyle) 6 wk after transformation; bar = 0.3 cm. **(B)** Axenic culture of hairy roots of *C. arabica* cv Caturra; long-term maintenance in presence of IBA in the dark; bar = 10.5 mm. **(C)** Histochemical localization of β-glucuronidase (GUS) gene expression in transgenic roots of *C. arabica* transformed with the

fresh media (Yasuda medium for *C. canephora* and T2B medium for *C. arabica*) in a 5.5-cm Petri dish. Incubate the culture under low light (10 µE/m^2/s, 16-h/d), at 27°C.

2. Three wk before transformation, transfer coffee callus cultures from *C. canephora* or *C. arabica* genotypes to the T3B medium with appropriate density (1 g/50 mL of semi-solid medium on a 10-cm diameter Petri dish with a 50-µm nylon mesh). Keep at low light (10 µE/m^2/s), for a 16-h photoperiod, at 27°C.

3.1.4. Agrobacterium tumefaciens *Culture Preparation*

1. Prepare the *A. tumefaciens* culture from a −80°C glycerol stock (*see* **Notes 7** and **8**). Inoculate bacteria from the 20% glycerol stock to 25 mL of YEP liquid medium with appropriate antibiotics: kanamycin 50 mg/L, rifampycin 50 mg/L, and eventually streptomycin 100 mg/L (for LBA) or gentamycin 20 mg/L (for C58). Supplement with acetosyringon at a final concentration of 100 µ*M*.
2. Grow the bacterial culture overnight on a shaker incubator (250 rpm, at 28°C) until the optical density (OD) reaches 0.3 to 0.5 (OD_{600nm}) (*see* **Notes 9** and **10**).
3. Centrifuge the bacteria (2500*g*, for 10 min), resuspend in a 25 mL of 0.9% NaCl solution supplemented with 200 µ*M* acetosyringon. It is not required to check OD at that point.

3.1.5. Infection and Co-cultivation

1. Submerge coffee calli in *A. tumefaciens* suspension (in NaCl and acetosyringon) for 2 h in 250-mL flasks, shaking constantly (100 rpm) (*see* **Notes 11** and **12**).
2. Gently eliminate the excess of bacterium culture by blotting briefly on sterile Whatman paper and co-cultivate coffee cells with bacterium on a 50 µm nylon mesh overlay on semi-solid Yasuda medium with 200 µM acetosyringon (10 cm Petri dish), at 27°C in the dark for 2 d.
3. After co-cultivation, rinse cells in liquid Yasuda medium supplemented with 1 g/L cefotaxime in 500-mL Erlenmeyer flasks for 4–5 h, shaking constantly (100 rpm). Use 50 mL medium and 0.1 g coffee calli per flask.
4. Gently blot dry coffee calli on sterile Whatman paper and culture them on semi-solid Yasuda medium supplemented with 400 mg/L cefotaxime on a 10-cm diameter Petri dish with a 50 µm nylon mesh. Keep at low light (10 µE/m^2/s), for a 16-h photoperiod at 27°C.

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p35S-*gusA-int* gene construct. The blue staining (⟶) allows the localization of tissues that actively express the reporter *gus* gene. The staining is strong in all the meristematic regions and central cylinders; bar = 10 mm. (**D–F**) Genetic transformation of *C. canephora* using *Agrobacterium tumefaciens*. (**D**) Embryogenic callus used for co-cultivation with *A. tumefaciens* is growing at the edge of a foliar explant, bar = 1.5 mm. (**E**) Transformed *C. canephora* plants of different ages after ex vitro acclimation: 0, 1, 3, 6 and 12 mo; bar = 50 mm. (**F**) Three years old field grown transgenic coffee plants in an insect resistance trial; bar = 150 mm.

3.1.6. Selection and Regeneration

1. After a period of 28 d, transfer cells to the same medium supplemented with selective agent (80–200 µg/L chlorsulfuron), in the same container, and under the same light conditions as above (*see* **Notes 13–15**).
2. Sub-culture cells every 4 wk by a simple transfer of the nylon mesh with the cells from the old medium to a fresh medium. This sub-culture is necessary because of the decreasing efficiency of the herbicide under culture conditions. Incubate the plates under low-light conditions (10 µE/m^2/s), with a 16-h photoperiod at 27°C (*see* **Note 16**).
3. Three months after co-culture, remove cefotaxime, but still supplement medium with selective agent.
4. After 3 to 8 mo on the selective medium, small (potentially transgenic) calli or embryos appear. Sub-culture these calli onto a new medium without mesh, with a selective agent for easier regeneration. Place Petri dishes under direct light (45 µE/m^2/s), with a 16-h photoperiod (*see* **Note 17**).
5. Cultivate young (torpedo to cotyledonary) embryos on a MS germination medium under direct light for 2 to 3 mo, with transfers to fresh medium every 4 wk.
6. After development (*i.e.,* when both cotyledonary leaves are spread), transfer the embryos to the rooting medium for 2 to 3 mo, with transfers to fresh medium every 4 wk.
7. Acclimate small plantlets in the greenhouse. First, cut out in vitro non-functional roots and then basal end of the stem in a commercial rooting powder (IBA) and transfer to a sand–soil mixture in small (50 × 30 × 30 cm) glasshouses (Puteaux S.A., Le Chesnay, France) at a very high hygrometry.
8. Water when needed by spraying a fog-type cloud with tap water. Start reducing humidity after 5 d by lifting the cover of the glasshouse, progressively increasing the opening over a period of 2 wk; remove the cover after acclimation
9. After 1 to 2 mo transfer to small pots with standard horticultural soil mixture and grow in a greenhouse (*see* **Fig 1E**). Use a 12-h photoperiod and a temperature between 26°C and 30°C as standard conditions.
10. Transgenic coffee plants are maintained the same way as control plants, fertilized when needed, under a natural light intensity; 12-h photoperiod and temperature between 26°C and 30°C. Plants are not brought to flowering, because only leaves are needed for molecular studies.

3.1.7. Molecular Analysis and Field Study

1. GUS histochemical assay: Incubate calli, shoots, leaves, or roots overnight at 37°C in the classical medium defined by Jefferson ***(44)***, modified by the use of phosphate buffer (0.2 *M*, pH 7.0). Add methanol (20% v/v) to eliminate any eventual endogenous or interfering expression of the non integrated GUS gene.
2. Polymerase Chain reaction (PCR) and Southern analysis are performed using DNA from coffee leaves to verify the transgenic nature. Genomic DNA extraction is performed using a Qiagen® kit.

3. Transfer a sample of the plants to the field for an agronomic and resistance evaluation *(45)*, (*see* **Fig.1F**). A 5-yr experiment is necessary to obtain reliable results and confirm the expression of the gene introduced during the 3 first harvests of the trees (*see* **Note 18**) (*34,46,47*).

3.2. Hairy Root Regeneration Using A. rhizogenes

3.2.1. Seed Sterilization and Zygotic Embryos Germination

1. Seed sterilization: Remove the parchment from the coffee beans by hand. Sterilize by immersing the beans in seed surface disinfectant solution. Stir for 5 min, apply a vacuum for 20 min, and then stir for 5 min. Rinse 3 times in sterile water.
2. Soaking: Divide the seeds up into 10-cm diameter Petri dishes (2-cm deep), containing sterile water, and place in the dark at 27°C. Under these conditions the seeds will be totally imbibed after 48 to 72 h.
3. Embryo extraction: Remove the pergamine. Use a scalpel to remove the endosperm over the embryo, cutting from the root pole towards the cotyledons. Then extract the embryo levering it out from the root pole with the same scalpel blade.
4. Germination: Culture the zygotic embryos in 5.5-cm diameter Petri dishes (3 embryos/dish and 12.5 mL of medium) on semi-solid GER medium. Place the dishes in the dark at 27°C for 8 wk. At the end of that period the embryos will have started germinating; they will have a 12-mm long hypocotyl and a root about 10-mm long.

3.2.2. Transformation and Hairy Root Induction

1. Grow the *A. rhizogenes* strain (with the appropriate construct) from a −80°C 40% glycerol stock on MYA semi-solid medium with appropriate antibiotics. The bacteria should be grown at 28°C for 48 h to be used directly for genetic transformation.
2. Use a scalpel with a blade contaminated by drawing it over the bacterium culture.
3. Make a 2-mm long wound in the hypocotyl of the zygotic embryos with the contaminated blade.
4. Culture the wounded embryos in 5.5-cm diameter Petri dishes (3 embryos/dish) containing sucrose-free GER medium. Place the dishes in the dark at 18°C for 2 wk (*see* **Note 19**).
5. After co-culturing, rinse the embryos for 2 h in liquid GER medium containing 500 mg/L of cefotaxime. Cut the taproot well above the collar and discard (*see* **Note 20**).
6. Culture the infected embryos (3 embryos/5.5-cm dish) on GER medium with 500 mg/L cefotaxime under low light (10 $\mu E/m^2/s$), with a 14-h photoperiod at 27°C for 4 wk. Transgenic roots will appear after 3 wk at the wound and bacterium inoculation site.
7. Transfer the embryos (3 embryos/dish) to 2.5 × 10-cm Petri dishes containing GER medium + 250 mg/L cefotaxime under same incubation conditions for 4 wk. Several 4- to 6-cm long roots will develop from the inoculation site (*see* **Fig.1A**).

8. Culture the embryos again on GER medium + 150 mg/L of cefotaxime (2 embryos/dish) under same conditions for another 4 wk.
9. At this time, the transgenic roots (hairy roots) will be highly branched and can be sectioned (*see* **Note 21**). They should then be grown in the dark on GER medium supplemented with 2.5 μM of IBA in 10-cm diam Petri dishes (*see* **Note 22**). The culture can be maintained for long period by fresh transfers to this nutrient medium every 4 wk at 27°C (*see* **Fig. 1B**).

3.2.3. Molecular Analysis of Hairy Roots

1. Histochemical GUS assay (*see* **Fig. 1C**): To assay GUS activity, drench sectioned hairy roots with a staining solution containing 1 m*M* 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc), and incubate overnight at 37°C, as indicated by Jefferson ***(44)***. To confine the localization of the blue staining, add 0.5 m*M* $K_3Fe(CN)_6$ and 0.5 m*M* $K_4Fe(CN)_6$ as catalysts.
2. DNA extraction from hairy roots: For DNA isolation from root tissue, 1 g fresh weight can be either lyophilized or ground under liquid nitrogen into a fine powder using a mortar and pestle. Transfer the tissue powder to a 2-mL tube. Lyophilized samples can be store a 4°C and disrupted at ambient temperature, while frozen samples should be store a –80°C. When grinding plant tissue, samples tubes should be kept in liquid nitrogen and the sample not allowed to thaw. DNeasy® Plant Mini Kit N 69104 (Qiagen®) showed to be an appropriate procedure to obtain adequate purified DNA concentration (>20 μg) required for following molecular analysis.
3. PCR analysis: Use hairy roots that display a positive reaction to the GUS histochemical test. For amplification of a 584-bp fragment of the *gus* (*uidA*) gene use primers 5′-GAATGGTGATTACCGACGAAA-3′ and 5′-GCTGAAGAGATGCTC GACTGG-3′. The PCR mixture should consist of 5 μL (5 ng) of plant DNA, 2.5 μL of 10X Taq buffer (Promega), 1.5 μL of 25 m*M* $MgCl_2$, 1.0 μL of 5 m*M* deoxynucleotide triphosphate (dNTP), 0.25 μL of Taq DNA polymerase (5 U/μL Promega), 1 μL from each 10 pmol primer, and 12.75 μL of sterile distilled water. Perform PCR analysis with a PTC-100 Programmable Thermal Controller (MJ Research Inc., San Francisco, CA). Heat samples to 94°C for 5 min, followed by 29 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min, and then 56°C for 10 min. Separate amplified products by electrophoresis on 1.0% agarose gels with 0.5 mg/L ethidium bromide in 0.5X TAE and detect by fluorescence under ultraviolet light.

4. Notes

1. Tests showed that this methodology also worked with somatic embryos and could therefore be used on heterozygous materials.
2. Screenable marker: We used the *uidA* bacterial gene isolated from *E. coli* coding for β-glucuronidase with an additional intron for specific expression in plants ***(43)***. The gene was controlled by the cauliflower mosaic virus (CaMV) 35 S promoter and terminator.

3. Selectable marker: We used the *csr1-1* gene isolated from *Arabidopsis thaliana* (*48*) conferring resistance to the herbicide chlorsulfuron. This herbicide was used for selection of transformed cells. The gene was controlled by the CaMV promoter 35S with a duplicated enhancer sequence (*49*), and by the *csr1-1* terminator.
4. Trait gene: We used the *cry*1Ac gene from *B. thuringiensis* in a modified form synthetized at the University of Ottawa (*50*). After preliminary studies on coffee cells demonstrating its efficiency in transient expression (*51*), the *EF1α* promoter from *Arabidopsis thaliana* (*52*) was chosen, together with a promoter enhancer sequence Ω′ derived from tobacco mosaic virus (*53*) and the nopaline synthase terminator.
5. Phytagel™ was used at a dose of 6 g/L for Yasuda because the salt composition of this medium is quite low. In order to gel and form a matrix, Phytagel™ needs a minimum level of salts.
6. Five *Agrobacterium rhizogenes* wild strains were compared for transformation efficacy: 1583 also called A4 (agropin mannopin strain), ARqua1 (agropin mannopin strain), 1724 (mikimopin strain), 2659 (cucumopin strain) and 8196 (mannopine strain). The A4 strain was the most efficient (80% transformation efficacy), then ARqua1 (30%), 1724 (10%), and 2659 (5%). Hairy roots were not obtained with the 8196 strain.
7. Different *A. tumefaciens* strains can be used for coffee transformation. Based on previous results on other plants, LBA4404 was first used to produce transgenic plantlets. Later, two C58 strains (GV2260 and PMP90) have were used with similar results. Recently, EHA105 strain has also been used and achieved better results on coffee cells.
8. To prepare 20% glycerol culture mix 80% autoclaved glycerol solution with 0.25 (v/v) volume of bacterial culture of $OD_{600} = 1.5$. Deep freeze in −80°C.
9. Both YEP or LB (10 g/L bacto-tryptone, 10 g/L yeast extract) medium can be used for bacteria growing. However, *Agrobacterium* grows slightly slower in Luria-Bertani (LB) medium than in YEP medium.
10. Several binary plasmids have been used for coffee transformation. pBin19 was the first used, and allowed regeneration of transformed plantlets for all the studied genotypes. Nevertheless, pCambiaA1, a vector that was recently used to make new constructs, appears to be more efficient for transformation.
11. As described (*24*), somatic embryos could also be transformed by *Agrobacterium*. They have to be wounded with a scalpel before soaking in the bacterium suspension. Afterwards, they are cultivated like cells, and transformed calli or embryos will appear on these primary explants. It is difficult to obtain a large number of embryos at the correct stage (late torpedo), and the rate of transformation always remains low (under 10%).
12. Soaking of cells with bacteria can be done in liquid MS medium without hormones or directly in bacteria medium.
13. Chlorsulfuron has been the main herbicide used for selection of transformed events. Other selective agents have been used, such as bialaphos. The constructs with the *bar* gene, conferring resistance to this herbicide, were designed. Unfortunately, the

bar gene used in our constructs was not a synthetic gene, and performed poorly in our experiments. Hygromycin has also been used for coffee transformation (*22*). If kanamycin is used, it should be used at high concentrations (more than 400 mg/L). An alternative approach involving xylose isomerase gene (*xylA*) as a positive selection marker has been recently developed on coffee (*54*).

14. During selection pressure with the herbicide, coffee calli were cultured on Yasuda medium. For some *C. arabica* genotypes i.e., fixed lines it could be better to grow cells on a slightly richer medium, like half-strength MS.
15. The working dose for selection on chlorsulfuron can be variable, depending on genotype used. For *C. canephora* and Catimor genotypes, the classical dose of 80 μg/L is effective. For some *C. arabica* genotypes, i.e., hybrid varieties, a higher dose of up to 200 μg/L is necessary.
16. Culture in liquid medium in flasks instead of semi-solid medium for cell culture after co-cultivation could be used. Some transformation events have been obtained with this method, but it remains difficult to control growth of transformed cells, and to avoid mixtures of different transformation events.
17. The transformation efficiency is variable from one genotype to another. For *C. canephora* genotypes, the efficiency raises up to 50% of transformed calli growing from transformed cells—we defined the efficiency as the percentage of co-cultivated calli with a secondary transgenic callus growing. For *C. arabica* genotypes, the efficiency is lower, up to 30%. The second critical step is the regeneration of transformed embryos from callus. That is why cefotaxime has to be removed as soon as possible, and transformed calli have to grow on the selective medium at a very low density.
18. Some of the first experiments were conducted with the armed *A. rhizogenes* A4 using the same protocols as *A. tumefaciens* strains. Some plants have been regenerated from transformation with *A. rhizogenes*, but they never developed in the field.
19. The temperature during the coculturing stage had a marked effect on transformation efficiency with *A. rhizogenes*. For example, the results obtained were 70 to 80% for embryos transformed at 18°C and 20 to 30% at 27°C (*55*).
20. We discovered competition in the growth of the two types of roots. Eliminating the nontransgenic taproot encouraged the development of transgenic roots.
21. Hairy roots must be sufficiently developed before being sectioned for them to develop autonomously.
22. Coffee tree hairy roots need an exogenous supply of auxin to grow. The nature and concentration of the auxin affect their development.

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Papaya (*Carica papaya* L.)

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Summary

Transgenic papaya plants were initially obtained using particle bombardment, a method having poor efficiency in producing intact, single-copy insertion of transgenes. Single-copy gene insertion was improved using *Agrobacterium tumefaciens*. With progress being made in genome sequencing and gene discovery, there is a need for more efficient methods of transformation in order to study the function of these genes. We describe a protocol for *Agrobacterium*-mediated transformation using carborundum-wounded papaya embryogenic calli. This method should lead to high-throughput transformation, which on average produced at least one plant that was positive in polymerase chain reaction (PCR), histochemical staining, or by Southern blot hybridization from 10 to 20% of the callus clusters that had been co-cultivated with *Agrobacterium*. Plants regenerated from the callus clusters in 9 to13 mo.

Key Words: Embryogenic plant cell cultures; *Carica papaya* L. Hawaiian solo cultivars.

1. Introduction

Papaya (*Carica papaya* L.) trees are grown for fruit and papain, a commercially valuable proteolytic enzyme. Papaya is one of the few plant species which fruit throughout the year and can produce ripe fruit in as little as 9 mo from planting. A papaya tree may live for 25 yr or longer, bearing continuously with one or more fruit in each leaf axil and each fruit containing about 1000 seeds. Hand pollination is easily accomplished by transferring pollen from anthers of male or hermaphrodite flowers to the stigmata of female or hermaphrodite flowers. Having continuous flowering throughout the year, coupled with the ease of making crosses that produce a large number of progeny and fairly reliable genetic transformation make papaya an attractive model for genetic/genomic research and crop improvement.

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The scientific breakthrough in developing papaya as the first genetically engineered commercial fruit crop in the United States is well known. Papaya was transformed *(1)* with the coat protein gene of *Papaya ringspot virus* (PRSV) to produce a breeding line and cultivars with complete resistance to specific strains of PRSV. Subsequently, papaya was transformed for PRSV resistance by a number of groups *(2–7)* using gene transfer with *Agrobacterium tumefaciens*. The tissues transformed by *Agrobacterium* included leaf discs and petioles but, most commonly used were embryogenic tissue cultures. The highest levels of *Agrobacterium* transformation were obtained by co-cultivation following wounding of embryogenic tissues with carborundum or tungsten. Selection of transformed lines for resistance to kanamycin (generally at 150 mg/L) was accomplished in 6 to 13 mo. Regenerated plants generally had a single-gene insertion and were resistant to PRSV. Recently, papaya was transformed *(8)* with a particle gun using an antifungal resistance gene to improve papaya resistance to the oomycete pathogen *Phytophthora palmivora.* Transgenic papaya lines may become the basis for increased papaya fruit production worldwide after deregulation of its genetically modified (GM) status.

2. Materials

2.1. Plant Materials

Papaya seeds cv. 'Kapoho' (Hawaii Agriculture Research Center).

2.2. Bacterial Strains

1. *Escherichia coli* strain: MAX Efficiency DH5α™ chemically competent cells (Invitrogen, www.invitrogen.com).
2. *Agrobacterium* strains: disarmed laboratory strains, LBA4404 (Invitrogen, www.invitrogen.com) and EHA105 *(9)*, containing an engineered binary transformation vector based on pBI121 vector (Clontech, www.clontech.com) (*see* **Note 1**).

2.3. Media

1. YEP medium: 5.0 g/L Bacto-yeast extract, 10.0 g/L bacto-peptone, 10 g/L NaCl, 15 g/L bacto-agar, pH 7.2.
2. Callus induction medium: Half-strength Murashige and Skoog (MS) salts medium *(10)* (Gibco, www.lifetech.com) solidified with 2.5 g/L (0.25% w/v) Phytagel (Sigma), supplemented with 100 mg/L *myo*-inositol, 70 g/L (7%, w/v) sucrose, and full strength MS vitamins (0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine·HCl, 0.1 mg/L thiamine·HCl, and 2 mg/L glycine) and 10 mg/L 2,4-D, pH 5.6 to 5.8.
3. Callus selection medium: Identical to the callus induction medium but contains selective compounds as appropriate (*see* **Note 2**).

4. MBN shoot regeneration medium: MS medium supplemented with 2 mg/L benzylaminopurine (BA), 2 mg/L napththalene acetic acid (NAA), and solidified with 2.5 g/L (0.25%, w/v) Phytagel, pH 5.6 to 5.8.
5. Indole butyric acid (IBA) rooting medium: MS medium supplemented with 2 mg/L BA, 2 mg/L indoleacetic acid (IAA), and solidified with 2.5 g/L (0.25%, w/v) Phytagel, pH 5.6–5.8.

2.4. Reagents, Solutions and Other Supplies

1. 2,4-Dichlorophenoxyacetic acid (2,4-D) stock solution (1 mg/mL): Make 100 mL of 2,4-D stock solution by weighing 100 mg of 2,4-D (Sigma) and dissolving in minimum 1 *N* NaOH or 95% (v/v) ETOH adding 3 to 5 mL of solvent drop wise (*see* **Note 3**). Deionized water is added to make a total volume of 100 mL and is sterilized by passing the solution through a syringe filter. The stock solution can be stored at 4°C.
2. Carbenicillin disodium salt or cefotaxime sodium salt (100 mg/mL): Dissolve 100 mg of carbenicillin or cefotaxime (Agro-bio, www.agri-bio.com) into 100 mL of deionized water and sterilize through a syringe filter; aliquot into sterile 2 mL tubes and store at −20°C.
3. Geneticin disulfate salt, G418 stock (100 mg/mL): Dissolve 100 mg of G418 (Agro-bio; www.agri-bio.com) into 100 mL of deionized water and sterilize through a syringe filter, aliquot into sterile 2-mL tubes and store at −20°C.
4. Acetosyringone (3,5-dimethoxy-4-hydroxyacetophenone) (Sigma-Aldrich; www.sigma-aldrich.com): Prepare 0.3 *M* stock solution and store at −20°C, use aliquot into *Agrobacterium* solution at 20 μ*M* final concentration.
5. GUS staining solution: 50 m*M* $NaHPO_4$ at pH 7.2, 0.5% (v/v) Triton X-100, 1 m*M* X-Gluc (5-bromo-4-chloro-3-indolyl-beta-D-glucuronide cyclohexyl ammonium salt [USB Corporation] diluted from a 20 mM stock made up in dimethylformamide).
6. Tris ethylene-diamine tetraacetic acid (EDTA) (TE) buffer: 100 m*M* Tris-HCl, pH 8.0 and 10 m*M* EDTA, pH 8.0.
7. Tris-acetate-EDTA electrophoresis (TAE) buffer: 40 m*M* Tris-acetate and 1 m*M* EDTA.
8. Extraction buffer for genomic DNA (300 mL): 30 mL of 1*M* Tris-HCl at pH 8.0, 30 ml of 0.5 *M* EDTA at pH 8.0, 30 mL of 5 *M* NaCl, 18.7 mL of 20 % (w/v) sodium dodecyl sulfate (SDS), use deionized H_2O to adjust final volume to 300 mL.
9. Purifying buffer: 70% (v/v) EtOH, 0.3 *M* NaOAc.
10. Prehybridization buffer for Southern blot Analysis: 7% (w/v) SDS, 1% (w/v) bovine serum albumin (BSA) (Sigma), 1 m*M* Na_2EDTA, and 0.25 *M* Na_2HPO_4, pH 7.4.
11. First washing buffer for Southern blot analysis: 0.5% (w/v) BSA; 1 m*M* Na_2EDTA; 40 m*M* Na_2HPO_4, pH 7.4; and 5% (w/v) SDS.
12. Second washing buffer for Southern blot analysis: 1 m*M* Na_2EDTA; 40 m*M* Na_2HPO_4, pH 7.4 and 1% (v/v) SDS.
13. Potting media: Sunshine mix 4 (Horticulture Supply Company, HI)
14. Slow release fertilizer: Osmocote 14-14-14 (Scotts; www.scottscompany.com).

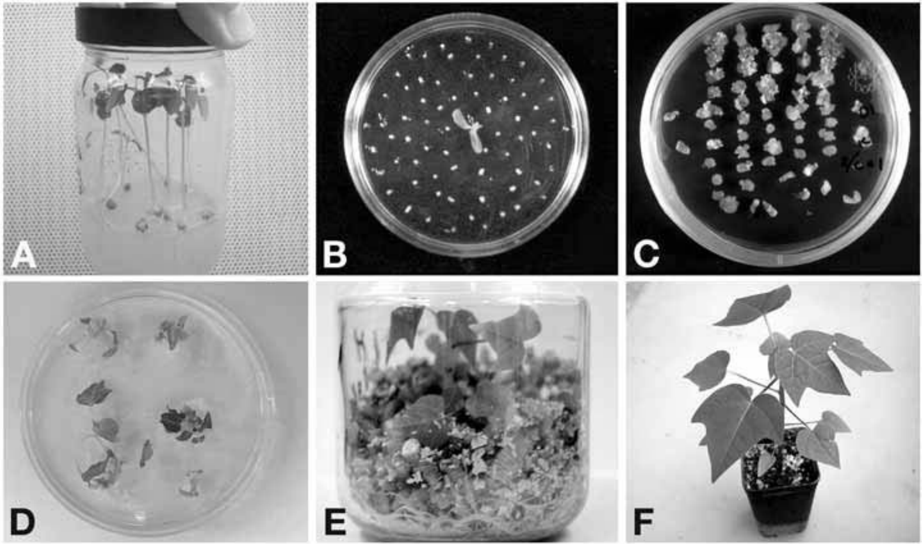

Fig. 1. Papaya regeneration from 2,4-D-induced somatic embryogenesis in hypocotyls sections. **(A)** 2-wk-old papaya seedlings germinated on 1% water agar. Hypocotyls and other tissues were explanted from seedlings at this stage of development. **(B)** Explanted hypocotyl sections 1- to 2-mm long on callus induction medium containing 10 mg/L of 2,4-D. **(C)** Hypocotyl sections on callus induction medium containing 10 mg/L 2,4-D after 2 mo in culture. All sections had developed into highly embryogenic calli. **(D)** Papaya plantlets regenerated from putative transgenic embryogenic calli via *Agrobacterium*-mediated transformation after 2 mo on MBN medium containing 2 mg/L BA and 2 mg/L NAA. **(E)** Papaya plantlets were rooted in IBA rooting medium containing the mixture (1:1[v/v]) of vermiculite and MS medium with 2 mg/L IBA, after 1 mo in culture. **(F)** Putative transgenic papaya plants in soil mix after harden in the lab.

3. Methods

3.1. Initiation of Somatic Embryogenic Calli From Hypocotyl Sections (11)

1. Sterilize seeds of papaya cv. 'Kapoho' (*see* **Note 4**) in batches of 50 by suspending them in 50 mL of 1.1% sodium hypochlorite for 1 h. Discard seeds with dark, unbleached spots or holes.
2. Rinse seeds in sterile distilled H_2O and shake for 24 h in 50 mL of 1 *M* KNO_3. Discard floating seeds and suspend the remaining seeds in 100 mL of sterile H_2O for about 5 d of imbibition with agitation at 32°C until the testae crack.
3. Sow germinating seeds on 1% (w/v) water agar and grow under cool white fluorescent lights (photosynthetically active radiation, PAR = 35 μmol/m^2/s) at 24 to 26°C for 2 wk or longer.
4. Seedlings showing expanded cotyledons and the first 2- to 4-cm long trilobed leaf at about 2 wk are used as explants (*see* **Fig. 1A**). Section the 3- to 12-cm-long

hypocotyls into 2- to 3-mm lengths and plate them on callus induction medium (*see* **Fig. 1B**).

5. Incubate the culture in the dark at 27°C for 6 to 8 wk until embryogenic calli can be observed growing on the hypocotyl segments. A majority of the hypocotyl sections will produce embryogenic calli over the following 2 wk.
6. Subculture the embryogenic calli on fresh induction medium every 3 wk to increase the quantity (*see* **Fig. 1C**).

3.2. Growth of Agrobacterium *Cultures and Preparation of Inoculum*

1. Transfer 5 mL of YEP liquid medium containing the appropriate selective antibiotics into a 20 mL culture tube (*see* **Note 5**).
2. Inoculate the tube containing YEP medium with a single colony of *A. tumefaciens* containing a binary vector with the gene of interest from a fresh plate (*see* **Note 6**).
3. Grow the bacterial suspension for 20–24 h at 28°C with agitation of 100 to 150 rpm until the culture appears turbid.
4. Determine the optical density of the bacteria cultures spectrophotometrically at 600 nm. The OD_{600} should be greater than 1.0 for the overnight culture.

3.3. Agro-infiltration and Co-cultivation (12,13)

1. Prior to transformation of papaya callus cultures with *Agrobacterium*, dilute the overnight-grown bacteria cultures with YEB medium in a flask to OD 0.5 to 1.0. Add 0.3 *M* acetosyringone (AS) to a final AS concentration of 20 μ*M*; mix well and continue to shake for at least 1 h before transferring into a 10-cm Petri dish for agro-infiltration.
2. Mix 0.5 g carborundum (600 mesh) and 20 mL induction medium in a 50-mL disposal centrifuge tube (Corning) with cap. Sterilize the suspension by autoclaving (*13*).
3. Add 1 g of papaya embryogenic callus clusters into the autoclaved carborundum suspension.
4. Vortex the calli on Super-Mixer at the speed setting of 6 or 7 for about 60 s.
5. Wash the calli two to three times with liquid callus induction media and withdraw the media using disposal syringes.
6. Immediately transfer the wounded the calli into the 10 mL *Agrobacterium* culture prepared in the 10-cm Petri dish. Keep the calli submerged in the suspension for 10 min.
7. Transfer the calli onto induction medium after blotting the excess *Agrobacterium* onto the sterilized paper towels. Co-cultivate in the dark at 24 to 26°C for 24 h.
8. Transfer the calli (about 20 pieces per plate) into fresh induction medium supplemented with 250 mg/L cefotaxime or 500 mg/L carbenicillin. If the *Agrobacterium* growth is excessive, wash the calli in the liquid induction medium containing 250 mg/L cefotaxime or 500 mg/L carbenicillin and blot with paper towels before transfer onto solid medium.

3.4. Selection of Transgenic Papaya Calli (12)

1. Transfer papaya calli onto the selection medium containing appropriate selective antibiotics (e.g., 100 mg/L G418 for *npt*II selection gene). Gently push the calli into the selection medium so the cells are in good contact with the selection agent in the solid medium.
2. Subculture the calli every 3 to 4 wk for a total selection period of 3 mo with cool white fluorescent light (PAR = 35 µmol/m^2/s) at 24 to 26°C.

3.5. Regenerating and Rooting of Transgenic Plants (11)

1. After 3 mo on the selection medium, transfer actively growing calli onto MBN regeneration medium.
2. Maintain the cultures with a 12-h cool white fluorescent light (PAR = 35 µmol/m^2/s):12-h dark cycle at 24–26°C.
3. Subculture the regenerating calli on MBN medium until the shoots (about 1-cm long) are formed (*see* **Fig. 1D**). Regeneration time is about 2 mo from subculture on MBN medium.
4. Separate the shoots from the clumps and transfer them to rooting medium for 1 wk in the dark at 24–26°C to initiate rooting.
5. Transfer the rooted shoots to vermiculite (30 mL) supplemented with half-strength Murashige and Skoog (MS) medium containing 2 mg/L IBA (30 mL) in Magenta boxes or baby food jars for rooting. Roots should be established in about 1 mo (*see* **Fig. 1E**).
6. Transfer the rooted plants to 4 × 4 inch pots containing a peat-based commercial potting mix and cover with clear plastic bags in a growth room with a temperature of 24 to 26°C and continuous cool white fluorescent lighting (PAR = 35 µmol/m^2/s) for one week. Plants are gradually acclimatized by increasing the opening of the bag about 10%/d. Plants are fertilized once/mo with small amounts of a complete slow release fertilizer.
7. Acclimatized plants (*see* **Fig. 1F**) are transferred to a greenhouse with temperature ranging from 20 to 26°C and photoperiod ranging from 11 h in winter to 13 h in summer. PAR in the greenhouse varied diurnally with the total daily maximum ranging from 35 to 50 moles/m^2/d. Approximately 90% of the transplants survive to become established.
8. Established plants, 12- to 20-cm tall, are transplanted to fields and grown with standard farm practices. Plant spacing is 1.5 m between trees within a row and 3 m between rows. Chemical NPK fertilizer (11:0:32) is broadcast around each tree at a rate of 20 kg/ha/mo for the first 3 mo and increased to 80 kg/ha/mo thereafter. Except for periods of high rainfall, plants are drip irrigated at the approximate rate of 100 m^3/ha/wk.
9. Field plants can be used for: collection of seed for genetic analyses (1000 seed/fruit and 1 fruit/wk) beginning at about 9 mo of age and continuous through 2 yr of age, and evaluation at various ages for the function of the transgene (e.g., if a disease resistance gene was transformed into papaya). The plants can be challenged with pathogen and evaluated for disease symptom expression.

3.6. Analyses of Transgenic Plants

1. Allow plants to grow sufficiently to provide enough tissue for analyses without compromising plant health; plants are assayed for transgene expression and for the presence of one or more transgene sequences.
2. Assays for the presence of the transgenes are conducted based on the transgene coding sequence. The PCR can be performed for early screening but DNA blotting (Southern blot) should be performed to determine the copy number and integration pattern of the transgenes.

3.6.1. GUS Histochemical Assay

1. About 3 wk following *Agrobacterium* infection, papaya tissue cultures can be examined for β-glucoronidase (GUS) expression using the histochemical assay (*14,15*).
2. Excise small callus or leaf sections and transfer them to microfuge tubes.
3. Fill the tube with the GUS staining solution but leave tops of tubes uncapped.
4. Remove air trapped in the tissue by placing the tubes in a bench top aspirator and pull a vacuum for 5 min or longer; release the vacuum and repeat. Be certain that the tissues are completely submerged in the solution.
5. Cap the tubes and incubate at 37°C with agitation for 24 h.
6. Remove the staining solution and replace with 70% EtOH.
7. Change the 70 % (v/v) EtOH twice over a 24-h period until chlorophyll is removed and the blue GUS stain is clearly visible.

3.6.2. Extract Total Genomic DNA From Fresh Papaya Leaves (see **Note 7**)

1. Add 1.1 g $NaHSO_3$ to 300 mL extraction buffer immediately before beginning the extraction process.
2. Grind fresh leaf tissue in liquid nitrogen. Add 20 mL extraction buffer to each tube of 10 mL (6–7 g) ground tissue. Mix well by vortexing. Incubate in a 65°C water bath for 1 h. Shake tubes at least once during incubation.
3. Add 6 mL of 5 *M* KOAc to each tube. Invert tubes gently several times until thoroughly mixed. Place tubes on ice for 20 min. Keep the samples cold either on ice or under refrigeration.
4. Centrifuge for 20 min at 2000*g*, at 4°C.
5. Label a set of new tubes and dispense 15 mL of cold (–20°C) isopropanol into each one. Pour the supernatant into the new tube through a layer of Miracloth. Do not mix the two phases and leave the tubes untouched at –20°C for 1 to 2 h.
6. Genomic DNA in the form of cotton threads can be seen at this stage. Hook out the DNA using a glass hook made from a glass pipet and transfer the DNA into a microfuge tube containing 1 mL purifying buffer. DNA can be stored in the purifying buffer at –20°C for several weeks at this stage.
7. Carefully pour off the purifying buffer.
8. Wash the DNA pellet in 1 mL cold (–20°C) 70% (v/v) EtOH for 1 min.

9. Carefully pour off EtOH. Air-dry the pellet until no odor of EtOH can be detected.
10. Add 200 to 300 μL of TE buffer to each tube. Leave tubes in a 4°C refrigerator until the DNA pellet dissolves. Quantify the DNA concentration using a spectrophotometer. Our typical yield is at least 100 μg DNA per 6 to 7 g of leaf material. Store DNA at −20°C.
11. DNA can be used for either PCR or Southern analysis.

4. Notes

1. Ti binary vector pBI121 can be purchased from Clontech (Palo Alto, CA). The pBI121 plasmid contains a neomycin-resistance gene (*npt*II) for selection of transgenic cells and a *gus* gene (*uidA*) for reporter protein expression. The *Agrobacterium* cells can be transformed with the pBI121 plasmid by electroporation using a Gene Pulser™ (Biorad; *www.biorad.com*).
2. Medium containing 300 mg/L of kanamycin or 100 mg/L of geneticin can be used to select transformants transformed with nptII gene.
3. 2,4-D doesn't dissolved in water, make sure it was dissolved in 1 *N* NaOH or 95% (v/v) ETOH completely before adding water.
4. The Hawaiian cultivar 'Kapoho' was the most reliable in producing embryogenic calli, but other cultivars, 'Sunrise', 'Sunset', and 'Waimanalo' also became embryogenic on the 2.4-D-containing media at a lower frequency. 'Kapoho' hypocotyls on induction medium containing 10 mg/L 2,4-D consistently produced high frequencies of embryogenic calli. Varying both the 2,4-D and sucrose concentration in the medium may increase the frequency of embryogenesis in cultivars other than 'Kapoho'. Addition of the growth regulator abscisic acid (ABA) may also increase the frequency of the embryogenesis (based on what?).
5. Rifampcin at 25 mg/L can be used for EHA105 and 25 mg/L of kanamycin can be used for LBA4404 plus 50 mg/L of kanamycin for selectable marker on the pBI121 vector.
6. To obtain active cultures of *Agrobacterium*, it is very important to use colonies actively growing on solid medium as an inoculum.
7. The DNA extract can be used for both PCR and Southern blot analyses. If the PCR method will be used, a small quantity of DNA can be extracted using the cetyl trimethy ammonium bromide (CTAB) method.
8. To perform several parallel reactions, prepare a master mix containing water, buffer, dNTPs, primers and Taq DNA polymerase in a single tube which can then be aliquoted into individual tubes. $MgCl_2$ and template DNA solutions are then added. This method of setting reactions minimizes the possibility of pipetting errors and saves time by reducing the number of reagent transfers.

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Pineapple [*Ananas comosus* (L.) Merr.]

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Summary

A procedure for pineapple [*Ananas comosus* (L.) Merr.] genetic transformation is described, which involves temporary immersion bioreactors (TIB) for selection of transgenic plants. Success in the production of transgenic pineapple plants combines tissue culture factors. Firstly, the use of regenerable pineapple callus as starting material for transformation whose cells shown to be competent for *Agrobacterium* infection. Secondly, the used of filtered callus, resulting in homogeneously sized clusters, thereby increasing the contact between the cell surfaces and *A. tumefaciens* and releasing phenolic compounds which induce *Agrobacterium* virulence. Thirdly, regeneration of primary plants without selection pressure, that allowing a massive production of putative transgenic pineapples. Finally, we support that TIB technology is a powerful system to recover nonchimera transgenic plants by micropropagation with the use of an adequate selection agent.

Key Words: *Agrobacterium tumefaciens*; transformation; pineapple.

1. Introduction

Pineapple (*Ananas comosus* (L.) Merr) is the third most important tropical fruit ***(1)*** and the cultivar Smooth Cayenne is preponderant worldwide ***(2)***. Among many stresses, fungal diseases (e.g., *Fusarium subglutinans* and *Phytophthora parasitica*) are important factors affecting the agronomic performance of this cultivar ***(3)***. Classical pineapple plant breeding is based on crosses, back-crosses, and selection ***(4)***. Because of the long generation cycle of pineapple, the conventional breeding programs are extremely time-consuming and can hardly keep pace with the rapid evolution of pathogenic fungi ***(5)***. Plant genetic transformation is an alternative strategy to save time and obtain fungus-resistant pineapple plants.

Transformation protocols have been set up for pineapple crop genetic modification and two patents have been published ***(6,7)***. Researches have been

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focused on manipulation of ethylene biosynthesis pathway to modify flowering and fruit ripening *(8)*, as well as disease resistance projects such as nematode *(9)* and virus control *(10)*, and production of fungi-tolerant cultivars *(11)*. Pineapple genetic transformation has been reported with the use of biolistics *(11,12)* and *Agrobacterium tumefaciens* *(11,13)*. Even though there are notices on pineapple genetic transformation, to our knowledge, this is the first protocol involving the use of temporary immersion bioreactors for pineapple transgenic plant production by *A. tumefaciens*.

2. Materials

2.1. Plant Material

Crown from elite pineapple (cv. Cayena Lisa Serrana) field grown plants *(14)*.

2.2. Agrobacterium tumefaciens Vectors and Strains

1. Vectors: For large-scale experiments, strains AT2260 (pHCA58) and AT2260 (pHCG59) were constructed with the aim to obtain further fungi tolerance in transgenic pineapples *(13)*. The plasmid pHCA58 contains a class-I bean chitinase (*chi*) gene under the control of a hybrid OCS-35S CaMV-rice actin I promoter (*pA5*) and the tobacco *ap24* gene under the 35S CaMV promoter. The plasmid pHCG59 contains the *chi* gene under the hybrid pA5 promoter and a class-I tobacco β-1,3-glucanase (*gluc)* gene under the 35S CaMV promoter. Both plasmids carry the *bar* gene for resistance to phosphirothricin (PPT) under the control of maize ubi promoter (*see* **Note 1**).
2. *Agrobacterium* strain: LBA4404 (pTOK233) and AT2260 (pIG121Hm) could also be used for methodological aims.

2.3. Culture Media (see Note 2)

1. Micropropagation medium (MM1): 4.4 g/L Murashige and Skoog (MS) salts (Duchefa Biochemie BV) plus vitamins *(15)*, 100 mg/L *myo*-inositol, 0.1 mg/L thiamine-HCl, 30 g/L sucrose, 0.6 g/L agar (Phytotechnology Lab), 1.0 mg/L 6-benzylaminopurite (BAP), and 1.0 mg/L α-naphthalenoacetic acid (NAA).
2. Multiplication medium (MM2): 4.4 g/L MS salts plus vitamins (Duchefa Biochemie BV), 100 mg/L *myo*-inositol, 0.1 mg/L thiamine-HCl, 30 g/L sucrose, 0.6 g/L agar (Phytotechnology Lab), 2.1 mg/L BAP, and 0.3 mg/L NAA.
3. Embryogenic callus formation medium (ECFM): 4.4 g/L MS salts plus vitamins (Duchefa Biochemie BV), 0.6 g/L agar (Phytotechnology Lab), 2.5 mg/L dicamba, and 0.5 mg/L BAP.
4. Plant regeneration medium (PRM): 4.4 g/L MS salts plus vitamins (Duchefa Biochemie BV), 100 mg/L *myo*-inositol, 0.1 mg/L thiamine-HCl, 30 g/L sucrose, 0.6 g/L agar (Phytotechnology Lab), 150 mg/L citric acid, and 0.5 mg/L BAP, with 200 mg/L cefotaxime (Phytotechnology Lab).
5. Selective propagation medium (SPM): 4.4 g/L MS salts plus vitamins (Duchefa Biochemie BV), 100 mg/L *myo*-inositol, 0.1 mg/L thiamine-HCl, 30 g/L sucrose,

2.1 mg/L BAP, and 0.3 mg/L NAA, with 200 mg/L cefotaxime (Phytotechnology Lab) and 2.5 mg/L phosphinotricin (Phytotechnology Lab).

6. Luria-Bertani (LB) medium: 10 g/L Bacto-tryptone (Becton Dickinson, Sparks, MD), 5 g/L yeast extract (Fisher Scientific, Suwanne, GA), 10 g/L NaCl (Fisher Scientific), 15 g/L bacto agar (Becton Dickinson, Sparks, MD). Autoclave, then cool to 55°C before adding the appropriate selective agent (dependent upon the vector).
7. *Agrobacterium* suspensions: 10 mL LB culture medium 10 mg/L bactotryptone, 5 mg/L bacto-yeast extract, 10 mg/L NaCl with 100 mg/L spectinomycin and 50 m*M* acetosiringone.
8. YEB medium: 400 mg/L yeast extract, 10 g/L mannitol (Fisher Scientific), 100 mg/L NaCl, 200 mg/L $MgSO_4 \cdot 7H_20$ (Sigma, St. Louis, MO). Autoclave, then cool to 55°C before adding the appropriate selective agent.

2.4. Stock Solutions and Other Supplies

All stock solutions listed below should be filter sterilized a 0.2-μ hydrophobic filter and stored in aliquot at 4°C.

1. Thiamine solution (1 mg/mL): Dissolve the powder in sterile distilled water (SDW).
2. BAP solution (1 mg/mL): Dissolve the powder in a few drops of 1 *M* NaOH. Make to final volume with SDW.
3. NAA solution (1 mg/mL): Dissolve the powder in a few drops of 1 *M* NaOH. Make to final volume with SDW.
4. Dicamba solution (1 mg/mL): Dissolve the powder in a few drops of 1 *M* NaOH. Make to final volume with SDW.
5. Spectinomycin solution (100 mg/mL): Dissolve the powder in SDW.
6. Cefotaxime: Use commercial (Phytotechnology Lab) Cefotaxime 500 (500 mg/mL).
7. Phosphinotricin solution (5 mg/mL): Dissolve the powder (Phytotechnology Lab) in SDW.
8. GUS assay buffer and solution: Prepare phosphate buffer mixing Na_2HPO_4 (100 m*M*) and $NaHPO_4$ (100 m*M*), pH 7.0. Add ethylene-diamine tetraacetic acid (EDTA) 10 mM, $K_4Fe(CN)_6 \cdot 3H_2O$ (0.05 m*M*), Triton X100 (0.1%). Dissolve X-Gluc powder at 1 mg/mL in the phosphate buffer. Filter-sterilize and use immediately.
9. Wizard Genomic DNA purification kit (Promega; cat. no. A1125).

3. Methods

3.1. Explant Preparation and Calli Culture Initiation

1. Collect crown from field-grown pineapple plants (*14*).
2. Carefully remove all the leaves from the crown; wash with detergent and flow water.
3. With a knife extract single buds of 1-cm^3 depth. Disinfect the buds with $HgCl_2$ 0.2%, for 3 minutes. Rinse five times with abundant SDW. Consider adequate health protection rules.

4. In a laminar flow bench, transfer disinfected explants to multiplication medium.
5. Shoots are sub-cultured at 45 d intervals for 3 mo at 25°C, 16-h light photoperiod, 125 $\mu E/m^2/s$ intensity.
6. For callus culture initiation, the three youngest and most internal leaves should be excised from in vitro pineapple shoots and cultured on embryogenic callus formation medium in the dark at 25°C. Five-mo-old calli can be used in transformation experiments.

3.2. Agrobacterium *Culture Preparation*

1. Grow *Agrobacterium tumefaciens* at 28°C for 48 to 72 h in Erlenmeyer's flasks containing 50 mL of YEB medium supplemented with adequate antibiotics.
2. When in exponential growth (OD_{600} = 1), centrifuge at 5300*g* and followed by suspending the pellets in 10 mL of LB medium.

3.3. *Calli Transformation*

1. The selection of embryogenic calli can be done with stereo microscope under laminar flow. The calli must be nodular and with an opaque yellow colour.
2. Before infection, the calli must be gently passed through 2000 μm mesh polypropylene filters (Spectrum). Place the sieved calli on Petri dish and dry for 10 to 15 min under laminar flow (*see* **Note 3**).
3. Transfer 2 g of calli to a Petri dish and add 2 ml of *Agrobacterium* suspension in exponential growth (OD_{600} = 1).
4. After 10 min, wash the infected calli 2 times for 1 min with distilled sterile water and dry in a sterile filter paper disk or sterile paper towel.
5. Transfer the infected calli to a semisolid (0.2 g/L agar), PRM medium for co-cultivation for 24 h (in the dark at 25°C) (*see* **Note 4**).
6. After completing the co-culture period, the calli must be transferred into a solid PRM medium supplemented with 200 mg/L of cefotaxime for an additional 4-wk period. Keep cultures at 25°C, 16-h light photoperiod, 125 $\mu E/m^2/s$ intensity. Transient GUS expression on infected calli can be evaluated during this stage (*see* **Note 5**). After 4 wk, emerging shoots could be observed at frequency of approximately 95%. This number of regenerated shoots should be considered to determine the transformation efficiency. Note that selection of resistant transgenic plants will made in a further propagation step by using TIB (temporary immersion bioreactor).
7. Regenerated shoots must be individualized and separate from nonregenerable pieces of calli. Subculture the plants under the same conditions above described until they are 5-cm size approximately.
8. Transfer the plantlets into a selective propagation medium (SPM), supplemented with 2.5 mg/L of phosphinotricin. The micropropagation system should be based on the automatic temporary immersion bioreactor (ATIB) (*see* **Note 6**). Shoots should be immersed in selective medium for 2 mins each for a total of 3 h. Maintain the cultures at 25°C, 16-h light photoperiod, 125 $\mu E/m^2/s$ for 4 wk.

9. The putative transgenic resistant plants must be multiplied in selective propagation medium (SPM). It must be taken into account that each clone is considered an independent transformation event. Keep at 25°C in 16-h light photoperiod 125 μE/m^2/s. Repeat 3 to 4 times the selective multiplication step in TIB for 60 to 90 d (*see* **Note 7**).

3.4. Growing Plants to Maturity

1. Take the plants out of the culture vessels (minimum fresh weight of 0.2 g) and wash gently with plenty of tap water.
2. Plant them in trays (V = 82 cm^3) containing a mixture of zeolite and filter cake (1:1, V:V). You can also use peat moss.
3. Place them in a greenhouse under an automated mist system. For the first 30 d the irrigation interval is kept constant each 30 min, while the irrigation time must be variable: 3 min for the first wk, 2 min in the second, and 30 sd in the last 2 wk. Maintain luminosity between 50 and 56.2 μE/m^2/s measured at noon, 16-h photoperiod. Functional roots should be directly induced during this period.
4. After 30 d the plants should be irrigated twice a day to saturate the substrate and the light is increased to 125 μE/m^2/s. You can also start using commercial fertilizers.
5. Maintenance of each transgenic clone should be by cutting the asexual shoots emerging from a single pineapple crown.

3.5. Molecular Analysis of Transgenic Plants

1. Using the automatic micropropagation system for transgenic pineapple plant selection in the present method may resulted in, recovering of chimera plants. Although the frequency of chimeric plants is low (5–6%) we suggest that molecular analysis must be performed to confirm the transgene integration in separate parts (roots, stems, leaves) of a putative clone.
2. Molecular analysis should be carried out according to your own cloning strategy for plasmid construction and following standard procedures as polymerase chain reaction (PCR) and Southern blot analysis for DNA integration, or Northern blot and reverse transcriptase (RT)-PCR to determinate the expression of transgenes ***(18)***.
3. For genomic DNA extraction, we typically use 2 to 3 g of leaf tissue from plants growing in greenhouse and immerse immediately in liquid N_2. Promega's Wizard Genomic DNA purification kit allows us to recover DNA with yield 0.8 to 1 μg/g of starting material. Two DNA extractions (corresponding to two individuals) per transgenic clone are recommended.

4. Notes

1. For methodological purpose, both LBA4404 (pTOK233) and AT2260 (pIG121Hm) could be used which have been previously described ***(13,16)***. The plasmids pTOK233 and pIG121Hm contain the kanamycin resistance gene (*npt*II) under the control of the *nos* promoter, as well as the hygromycin resistance gene (*hph*) and the GUS gene (*uidA)* both under the control of the ^{35}*S CaMV* promoter.

2. All culture media must be adjusted to a pH of 5.7 before sterilizing in an autoclave at 121°C and 1.2 kg/cm^2 for 15 min. The antibiotics and phosphynotricin stock solutions must be filter-sterilized through a 0.2 μ hydrophobic filter.
3. The desegregation of the embryogenic calli from the undifferentiated cluster using sieving step may have several benefits for *Agrobacterium*-mediated infection. It may improve the *Agrobacterium* infection ability because phenolic compounds must be released from broken calli to the medium. It may also increase the contact surface for *Agrobacterium* infection. However, because calli with lest than 1-mm size display low regeneration efficiencies, a compromise between transformation frequency and regeneration efficiency was obtained using pineapple calluses ranging 1.5- to 2.0-mm size; these showed an increase in the number of histochemical blue spots (GUS activity) detectable in comparison with the intact nonfiltered callus. Also, note that the sieved calli are air dried under laminar flow conditions for 10 to 15 min. The application of drying or osmotic treatments (15 min immersed in a Mannitol 3 *M* sterile solution) just before co-cultivation may favor penetration of *Agrobacterium* cells inside the calli clusters when re-hydrated during the infection period. These managements during tissue culture enable greater number of competent cells to be in direct contact with *Agrobacterium* and increase the transformation efficiency.
4. This is the optimum co-cultivation period to efficient interaction between *Agrobacterium* and genome of pineapple cells.
5. Optimization of the transformation conditions is necessary using different genotypes. In these cases, histochemical GUS assay ***(17)*** is recommended for monitoring the transformation efficiency. Take the samples (embryogenic calli) and put them over sterile paper disk imbibed with a sterile (1 mg/mL) X-Gluc solution. Incubate for 48 h at 28°C. Record the blue spots using a dissecting scope (Leica). Control treatments should be samples co-cultivated with LB culture medium.
6. Temporary immersion bioreactor (TIB) has been found to be an important tool for micropropagation of several crops allowing higher shoot formation rates and cost reduction. It seems to be, TIB increased contact between phosphinotricin and pineapple explants leading to a more dramatic reduction of proliferation rate in a highest selective conditions.
7. TIB system consisted of two containers; one for growing plants and a reservoir for liquid medium. The two containers are connected by silicone and glass tubes. In each case, the airflow is sterilized by passage through 0.2 μm hydrophobic filters. Air pressure from an air compressor pushed the medium from one container to the other to immerse the plants completely. The airflow is reversed to withdraw the medium from the culture container. Electronic timers controlled the frequency and length of the immersion period. Three-way solenoid valves provided on/off operation. The culture vessel dimension for conventional micropropagation is of 300 mL and two 300 mL vessels constituted the temporary immersion system. The culture medium reservoir included 150 mL of medium. Five plants are cultured in each vessel. Diagram of TIB system is shown in **Fig. 1**.

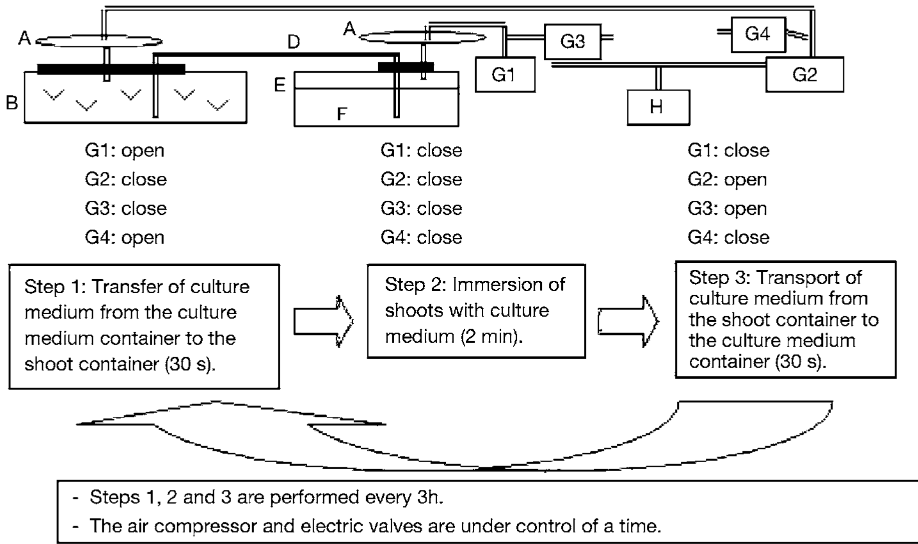

Fig. 1. Design of the temporary immersion bioreactor used for selection of pineapple transgenic plants. **(A)** Air filter (0.2 μm) (MIDISART 2000). **(B)** Glass vessel for shoots (300 mL). **(D)** Silicone tube. **(E)** Glass vessel for liquid culture medium (250 mL). **(F)** Liquid culture medium (250 mL). **(G)** Electric valve. **(H)** Air compressor.

8. Each single resistant plant must be under independent management; for instance, the use of separate recipient per putative transgenic clone during selective micropropagation is highly advisable. Frequently primary-regenerated plants die during the first selection because there is a high probability of recovering chimera shoots in the first propagation step. This probability is eliminated (or reduced) with successive propagations. The most promising plants will be those producing resistant shoots (clone). Those resistant plants that do not produce shoots in selective medium could be considered chimeras. Efficiency for recovering whole transgenic plants is about 5% (considering the total number of regenerated shoots) using TIB. Selection by conventional micropropagation could be tedious and efficiency for recovery of whole transgenic plants is reduced to between 1 and 2%.

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Sugarcane (*Saccharum* spp.)

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Summary

We describe the procedures for recovering transgenic sugarcane from co-cultivation of both calli and in vitro plants with *Agrobacterium tumefaciens.* The correct tissue culture strategies and the use of super-binary vector or super-virulent strain are crucial for the successful sugarcane transformation. Both plant regeneration via calli culture and micropropagation strategies can be optimized to a wide spectrum of sugarcane genotypes, thus the procedures presented here could be applied to genetic engineering of *Saccharum* spp. after minor modifications. For the case of sugarcane transformation using in vitro plants, four selective micropropagation steps must be sufficient to eliminate chimera plants.

Key Words: *Agrobacterium tumefaciens*; transformation; sugarcane.

1. Introduction

The soil plant pathogen *Agrobacterium tumefaciens* has become the most useful tool for plant transformation, including monocot species. *A. tumefaciens* is capable to transfer a particular DNA segment of the tumour-inducing (Ti) plasmid into the nucleus of infected cells where it is subsequently stable integrated into the host genome ***(1,2)***.

Sugarcane commercial varieties have been transformed by *A. tumefaciens* using embryogenic calli ***(3)*** and meristematic tissues from in vitro plants ***(4)***. Advantages of *Agrobacterium*-mediated transformation and approaches for increasing sugarcane cell competence to bacterial infection have been discussed ***(5,6)***. In summary, success in the production of transgenic sugarcane plants mediated by *Agrobacterium* has been attained by combining several tissue culture procedures, in particular: (i) The use of young regenerable material, characterized by the presence of actively dividing cells (competent for agroinfection), as it is the best for transformation experiments; (ii) pre-induction of the regeneration capacity, these include: (a) a pre-treatment step to

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improve the aeration conditions using culture shaking; (b) frequent changes of the culture media (supplemented with low auxin concentration), and (c) the use of short-time subculture period before co-cultivation and during the selection steps. (iii) Before infection, plant material should be carefully wounding to release phenolic compounds follow by a dried (or osmotic) treatment to improve the adhesion of bacterial cells during co-cultivation (rehydration). (iv) Co-cultivation of sugarcane materials with super-virulent *A. tumefaciens* or carrying super- super-binary vectors ***(7–10)*** is preferred to obtain the highest transformation efficiency.

In this paper, we provide details for the establishment of an efficient protocol for sugarcane transformation based on callus culture. Transformation efficiency using the callus culture system is about 0.5 to 1.5% (defined as number of hygromycin resistant plants per packed cell volume [PCV]) for cultivars Ja60-5 and C91-301. In addition, we also provide details of a more recent developed method using whole in vitro plants of sugarcane as starting material. Using the in vitro plant system, we can obtain transgenic plants with an efficiency of 3 to 5 hygromycin resistant clones/100 infect in vitro plants.

2. Materials

2.1. Plant Materials

Sugarcane cultivars: Modern cultivars Ja60-5 and C91-301 are commercial genotype products of recurrent introgressive hybridization resulting in highly heterozygous polyploids with a complex chromosome number, as has been demonstrated by genomic *in situ* hybridization ***(11)***. Sugarcane is an asexually propagated plant by stem (*see* **Note 1**). Starting material for transformation is derived from 6 to 9 mo old field grown plants.

2.2. Bacterial Strain and Plasmid

1. *A. tumefaciens* strain EHA101: carries a "dis-armed" version of pTiBo542 ***(7–10)***.
2. Vector pMTCA3IG: contains both *uidA* and *hpt* genes under the control of Pamy3 (α-amylase from rice) and 35SCaMV promoters, respectively ***(12,13)*** (*see* **Note 2**).

2.3. Culture Media

1. Callus induction medium P^{+5} (P^{+5}): 4.4 g/L Murashige and Skoog (MS) salts (Duchefa Biochemie BV) plus vitamins ***(14)***, 100 mg/L *myo*-inositol, 1 mg/L thiamine, 500 mg/L casein hydrolyzate, 5 mg/L 2, 4-dichlorophenoxy actic acid (2,4-D), 20 g/L sucrose, and 7 g/L agar, pH 5.6.
2. Callus induction medium P^{+1} (P^{+1}): Same as P^{+5} except supplemented with 1 mg/L 2,4-D.
3. Selective calli medium (SCM): Same as P^{+1} but supplemented with 30 g/L sucrose, plus 25 mg/L hygromycin, and 500 mg/L cefotaxime.

4. Selective regeneration medium (SRM): 4.4 g/L MS salts (Duchefa Biochemie BV) plus vitamins, 100 mg/L *myo*-inositol, 1 mg/L thiamine, 500 mg/L casein hydrolyzate, 30 g/L sucrose, 20 mg/L hygromycin, 500 mg/L cefotaxime, and 7 g/L agar, pH 5.6.
5. Shoot induction medium (SIM): MS salts, 0.2 mg/L 6-benzylaminopurine (BAP) 0.86 mg/L kinetin, and 30 g/L sucrose, pH 5.6. For a solid medium add 5 g/L agar.
6. Propagation medium (PM): MS salts, 1.3 mg/L indole-3-acetic acid (IAA) 0.86 mg/L kinetin, 0.3 mg/L BAP, and 30 g/L sucrose, pH 5.6. For a solid medium add 5 g/L agar. In case of selection add 20 mg/L hygromycin,
7. Selective rooting medium (SRM): MS salts, 1.3 mg/L IAA, 10 mg/L hygromycin, and 30 g/L sucrose, pH 5.6.
8. YEB medium (YEB): 5 g/L meet extract, 10 g/L yeast extract, 5 g/L peptone, 5 g/L sucrose, and 0.5 g/L $MgSO_4 \cdot 7\ H_2O$, pH 7.2.

2.4. Stock Solutions and Other Supplies

1. Antioxidant solution: Prepare 10 g/L ascorbic acid in sterile distilled water (SDW). Filter-sterilize and keep at 4°C.
2. BAP solution (1 mg/mL): Dissolve the powder in a few drops of 1 *M* NaOH. Make to final volume with SDW. Filter-sterilize and store at 4°C.
3. Kinetin solution (1 mg/mL): Dissolve the powder in a few drops of 1 *M* NaOH. Make to final volume with SDW. Filter-sterilize and store at 4°C.
4. IAA solution (1 mg/mL): Dissolve the powder in a few drops of 1 *M* NaOH or ethanol 50%. Make to final volume with SDW. Filter-sterilize and store at 4°C.
5. 2,4-D solution (1 mg/mL): Dissolve the powder in a few drops of ethanol 50%. Make to final volume with SDW. Filter-sterilize and store at 4°C.
6. Thiamine solution (1 mg/mL): Dissolve the powder in SDW. Filter-sterilize and keep at 4°C.
7. Hygromycin solution (25 mg/mL): Dissolve the powder in SDW. Filter-sterilize and keep at 0°C.
8. Cefotaxime solution: Use commercial Cefotaxime 500 (500 mg/mL). Filter-sterilize and keep at 0°C.
9. Spectinomycin solution (100 mg/mL): Dissolve the powder in SDW. Filter-sterilize and keep at 0°C.
10. Mixture soil and zeolite in 1:1 proportion.
11. Wizard Genomic DNA purification kit (Promega; cat. no. A1125).
12. β-glucuronidase (GUS) assay buffer and solution (1 mg/mL): Prepare phosfhate buffer mixing Na_2HPO_4 (100 m*M*) and $NaHPO_4$ (100 m*M*), pH 7. Add ethylene-diamine tetraacetic acid (EDTA) 10 m*M*, $K_4Fe(CN)_6 \cdot 3H_2O$ (0.05 m*M*), Triton X100 (0.1%). Dissolve 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) powder in the phosphate buffer. Filter-sterilize and use immediately.

3. Methods

3.1. Preparation of Agrobacterium *Culture*

1. Grow *Agrobacterium tumefaciens* at 28°C for 48 to 72 h in Erlenmeyer flasks containing 50 mL of YEB medium supplemented with adequate antibiotics. In the

case of EHA101 (pMTCA3IG), grow at 28°C for 48 h in YEB medium plus 100 mg/L spectinomycin.

2. When in exponential growth (OD_{600} = 1), centrifuge at 5300*g* and followed by suspending the pellets in 10 mL of YEB medium.

3.2. Preparation of Explants From Field Grown Material

1. Select the starting material (spindles) from 6 to 9 mo old field grown plants. Cut a section above the last visible dewlap for laboratory use. Basal zone of the stems with buds could be renewed as agamic seed.
2. Discard the mature leaves until reach a cylinder (primary explant) of approx 2-cm diameter of immature leaves of yellow-green colour.
3. Sterilize the primary explants by immersing in 70% ethanol for 3 min. Discard the liquid, and transfer the explants to a Petri dish in the laminar flow for sterilization. Immediately pass the flame from the gas burner to the Petri dish containing the explants by using a metallic instrument. Wait until flame is expired. Other sterilization methods can be tested.

3.3. Method 1: Callus Transformation

1. Discard the external immature leaves until reach a cylinder ranging from 0.5- to 0.8-cm diameter × 10-cm longitude. Cut the cylinder in pieces of approx 1 cm.
2. Place the explants on solid callus inducing media P^{+5}. Keep cultures in the dark at 25°C for 21 d (R_0 subculture).
3. Select the yellow and friable calli and transfer them to fresh calli inducing P^{+5} media at 25°C for 21 d (R_1 subculture).
4. Repeat the selection of the yellow and friable calli and transfer them to fresh P^{+5} media at 25°C for an additional 1 to 2 wk (R_2 subculture).
5. Select 2 g of calli, transfer them to an Erlenmeyer flask containing 50 mL of P^{+1} liquid media supplemented with 5 mL/L of antioxidant solution. Keep cultures in rotary shaker at 200 rpm in the dark for 7 to 10 d. Replacing the media every 3 d (*see* **Note 3**).
6. Discard the liquid medium. Calli should be filtered gently through sterile 1000 μm mesh polypropylene sieve (Spectrum) (*see* **Note 4**).
7. Air-dry the filtered calli under laminar flow for 30 min. In order to facilitate the manipulation, clusters should be plated on sterile filter paper disks of 2-cm diameter (*see* **Note 5**).
8. Transfer the disks to P^{+1} solid medium. Add 50 μL of *Agrobacterium* culture (OD_{600} = 1) on each paper disk. Keep in the dark at 28°C for 3 d for co-cultivation.
9. Wash the co-cultivated material three times with sterile distilled water. Dry in laminar flow bench for 10 to 15 min. Transfer the calli to sterile filter paper disks. Plate on P^{+1} solid medium supplemented with 500 mg/L cefotaxime. Keep 1 wk in the dark at 25°C.
10. Subculture to selective P^{+1} medium plus 25 mg/L hygromycin and 500 mg/L cefotaxime for 4 wk. Keep in the dark at 25°C. Control treatment should be ten filtered calli without *Agrobacterium* infection transferred to this selective medium.

11. Select the resistant calli and subculture in the same fresh selective medium for an additional 2 wk.
12. Transfer the putative transgenic calli to selective regeneration medium supplemented with 25 mg/L hygromycin and 500 mg/L cefotaxime. Keep at 25°C in 16-h light photoperiod (125 μE/m^2/s intensity).
13. After 3 to 4 wk, subculture the putative transgenic shoots of approx 0.5 cm to selective micropropagation medium plus 10 mg/L hygromycin and 500 mg/L cefotaxime.
14. Transfer the resistant plants to selective rooting medium supplemented with 10 mg/L hygromycin. Keep at 25°C, 16-h light photoperiod (125 μE/m^2/s) for 15 d.

3.4. Method 2: In Vitro Plant Transformation

1. Starting material selection and sterilization as described in **Subheading 3.2.**
2. Discard the external immature leaves until you reach a meristematic zone of approx 3-mm diameter × 10-mm longitude. Meristem should be carefully isolated and transferred to solid shoot-induction medium (SIM) (*see* **Note 6**). Keep at 25°C, 16-h light photoperiod (125 μE/m^2/s) for 21 d.
3. Subculture the regenerated shoot to solid propagation medium. Keep at 25°C, 16-h light photoperiod (125 μE/m^2/s) for 21 d.
4. Transfer the propagated plants to fresh liquid propagation medium for an additional 21 d. Repeat this step until multiplication rate be approx 1:5 (*see* **Note 7**).
5. Take high-quality (*see* **Note 8**) plant clusters and select those ranging 5 to 6-cm in size. Separate carefully each single plant producing little longitudinal wounds in the basal zones, dry under laminar flow for 15 min.
6. Immerse the basal zone of each plant in *Agrobacterium* culture (OD_{600} =1) for 10 to 15 min (*see* **Note 9**).
7. Blot infected explants over sterile paper disks to remove extra *Agrobacterium* culture in the laminar flow.
8. Transfer the plants to solid propagation medium. Keep at 28°C, 16-h light photoperiod (125 μE/m^2/s) for 3 d for co-cultivation.
9. Separately wash the basal zone of each plant in sterile distilled water three times. Dry in the laminar flow for 15 min.
10. Transfer to solid propagation medium plus 500 mg/L cefotaxime. Keep at 25°C, 16-h light photoperiod (125 μE/m^2/s) for 7 d.
11. Subculture the plants to solid propagation medium supplemented with 500 mg/L cefotaxime and 20 mg/L hygromycin. Keep at 25°C, 16-h light photoperiod (125 μE/m^2/s) for 21 d. Control treatment must be ten plants without *Agrobacterium* infection transferred to this selective medium.
12. Select the resistant shoots and transfer to selective propagation medium containing 30 mg/L hygromycin for 21 d. Repeat three times for eliminate chimeras plants (*see* **Note10**).
13. Transfer the resistant plants to selective rooting medium supplemented with 10 mg/L hygromycin. Keep at 25°C, 16-h light photoperiod (125 μE/m^2/s) for 15 d.

3.5. Growing Plants to Maturity

1. Following biosafety guidelines (could be case per case in different countries) (*see* **Note 11**), transplant each single resistant plant to a pot containing a mixture (1:1) of soil and zeolite.
2. Acclimate the transgenic plants in a controlled chamber with 85% humidity at 16-h light photoperiod (125 $\mu E/m^2/s$). Use solar filter if necessary to reduce excess of light. Adjust temperature to 28 to 30°C. Irrigation should be by spray three times/d for 10 min.
3. After 15 d, humidity could be reduced up to 65% and both photoperiod and light intensity should be according to natural environmental conditions. Irrigate by spraying two times/d for 10 min.
4. After 30 d and following appropriate biosafety guidelines transgenic plants should be ready to transplant for field trials.
5. Maintenance of each transgenic clone should be by cutting the stems containing buds as agamic seed.

3.6. Assay for GUS Activity

Optimization of the transformation conditions is necessary for different genotypes. In these cases histochemical GUS assay ***(15)*** is recommend for monitoring the transformation efficiency.

1. Take the samples (calli or basal zone of the in vitro plants) 7 to 10 d after infection and put it over sterile paper disk imbibed with a sterile X-Gluc solution. Incubate in the dark for 48 h at 28°C.
2. Record the blue spots using a dissecting scope (Leica). Control treatments should be samples that are co-cultivated with just YEB medium.

3.7. DNA Isolation

1. Cut approximately 5 g of leaf tissue from plants growing in greenhouse and immerse immediately in liquid N_2.
2. Use the Wizard Genomic DNA purification kit (Promega) for DNA extraction and purification. Typical DNA yield is 0.5 to 1 µg/gram of starting material. Two DNA extractions per sample are recommended.
3. Purified DNA is ready for Southern and polymerase chain reaction (PCR) analysis following standard molecular approaches ***(16)*** and according with your genetic construction.

4. Notes

1. Plants should be cultivated in a donor bank under optimal agronomic practices. Before used, genetic and phytopathologic quality should be evaluated by conventional and molecular methods to certify their identity and that they are free of major pathogens.
2. In sugarcane cells transformation, the usage of monocot promoters and the selection of transformed cells by hygromycin are highly advisable in order to achieve more efficient results ***(5,6,12)***.

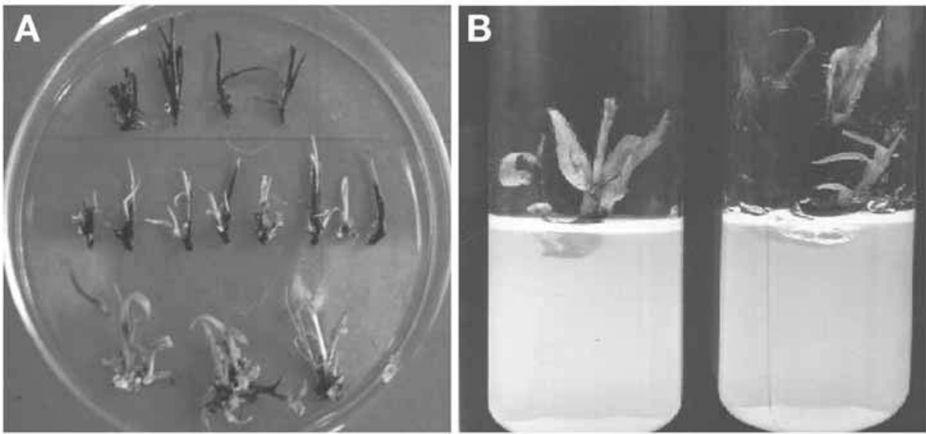

Fig. 1. Sugarcane transformation of in vitro plants by *Agrobacterium tumefaciens* infection. **(A)** First selection in micropropagation medium supplemented with 20 mg/L hygromycin. *Upper:* Noninfected plants subcultured to selective propagation medium (negative control). *Center:* Infected plants subcultured to selective propagation medium (treatment) showing some shoots growing from the basal zone of the original ones. *Lower:* Noninfected plants sub-cultured to nonselective propagation medium (positive control). **(B)** Second selection in micro-propagation medium supplemented with 30 mg/L hygromycin. Close up showing resistant shoots in selective micro-propagation medium. Note that the primary plant dies in the selective medium.

3. Determination of doubling time is advisable as it is dependent on genotype. The aim of this step is to reach actively dividing cells that are more susceptible to Agro-infection. Competent calli for transformation should be composed by a majority of small meristematic cells with large nuclei, thin cell walls and high viability.
4. This step improves the *Agrobacterium* infection ability because phenolic compounds must be released to the medium. An increasing of the contact surface for *Agrobacterium* infection is also obtained. We estimated that 1 mL of desegregated calli contains an average of 790 cell clusters 1000 µm size.
5. The application of drying or osmotic treatments just before co-cultivation favors penetration of bacterial cells inside the calli clusters when re-hydrated during the infection period. This process enables greater number of competent cells to be in direct contact with *Agrobacterium* and increases the transformation efficiency
6. Basal zone of the meristems must not be in close contact with the culture media in order to avoid phenolization. Culture tubes are recommends for this step.
7. Multiplication rate mean the number of plants asexually multiplied from a single plant. During the first steps, multiplication rates are relatively low (i.e., 2–3 plants from the original one). A high multiplication rate produces plants more competent for Agro-infection because cells are actively dividing. Plants from temporary immersion bioreactors (TIBs) displaying up to 1:80 rates are preferred for transformation experiments.

8. High-quality plants are considered as vigorous, healthy with minimum size ranging 5 to 6 cm. Plants displaying larger basal zones show better transformation efficiency because originate highest number of shoots. Wounds must be superficial in order to not affect the multiplication capacity.
9. For a further and efficient elimination of *Agrobacterium*, only the basal zone should be in contact with the bacteria culture. Excess of *Agrobacterium* should be avoided during co-cultivation.
10. During first selection majority of primary infected plants die in hygromycin medium (*see* **Fig. 1**). Plants after the second selection originate the following cases: (1) Not produce resistant shoot, (2) produce a single resistant shoot, (3) produce 2 to 4 resistant shoots. After three micro-propagation steps, shoots originated from a single infected plant material should be considered as independent event. According to our Southern blot analysis, after two propagation-selection steps the probability to recover chimera plants is approx 50%. This could be significantly reduced (or eliminated) after a fourth selective micropropagation.
11. Biosafety guidelines should be adequate for each country. In any case, rules must consider that acclimatization of transgenic clones should be in controlled greenhouses to avoid any disease or insect attack.

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V

Nuts and Fruits

22

American Chestnut [*Castanea dentata* (Marsh.) Borkh.]

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Summary

The key to successful transformation of American chestnut is having the correct combination of explant tissue, selectable and scorable markers, and a reliable regeneration system. Rapidly dividing somatic embryos, growing as proembryogenic masses, are a suitable tissue; the *bar* gene is a suitable selectable marker in conjunction with 1.0 to 10 mg/L phosphirothricin (PPT); and the *mgfp5-ER* gene is an effective nondestructive scorable marker. We have also found that the more gently the somatic embryos are treated during the inoculation and co-cultivation steps, the higher the transformation efficiency. The average transformation efficiency that can be expected using the described protocol is approx 20 stable and embryogenic transformation events/g of somatic embryo tissue. Cell line and batch-to-batch deviations both upward and downward should be expected. Finally, somatic embryos can be induced to form shoots, which can then be micropropagated and acclimatized.

Key Words: Desiccation; co-cultivation; somatic embryogenesis; *bar*; GFP.

1. Introduction

The American chestnut was one of the most important deciduous tree species in the eastern United States. Towering over most of the other trees, it often made up 25% or more of the overstory *(1)*. In 1904, the disease that became known as the chestnut blight was discovered in the Bronx Zoological Park *(2)*. Caused by the fungal pathogen *Cryphonectria parasitica* (Murr.) Barr., the chestnut blight has reduced this once magnificent species to little more than an understory shrub *(3)*.

The development of a reliable *Agrobacterium*-mediated transformation system will allow introduced disease resistance genes from outside the *Castanea* gene pool to help combat the blight. Research toward developing key elements of such a transformation system was carried out with both American and European chestnut (*Castanea sativa* Mill) throughout the 1980s and 1990s.

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Publications have been written about somatic embryogenesis ***(4–7)***, detecting transformation events using polymerase chain reaction (PCR) ***(8)***, testing marker genes ***(9–10)*** and on micropropagation ***(11–19)***. Further refinements were made to somatic embryo regeneration procedures for American chestnut by adding charcoal to the medium and including a chilling step ***(20)***. Asparagine has also been found to improve embryo conversion efficiency ***(21)***.

The first report of successful *Agrobacterium*-mediated transformation of a *Castanea* species described the regeneration of transgenic shoots from European chestnut cotyledons in 1998 ***(22)***. This was followed in 2004 by a report of the transformation of somatic embryos that were subsequently regenerated into whole plants through a combination of germination and micropropagation ***(23)***. We are unaware of any published reports of transgenic American chestnut whole plants.

A key step in the described protocol is the use of desiccation during co-cultivation ***(24)***. However, response to desiccation is genotype specific. We found clones that multiply primarily as heart-stage or torpedo-stage somatic embryos tolerate the described desiccation treatment without severe damage. Clones that multiply as tiny proembryogenic masses are more prone to damage from the desiccation treatment. For the latter clones, we devised another treatment which we call "plate flooding" in which the *Agrobacterium* inoculum is added to the embryos without moving them from the E1 medium. Using this plate flooding method we have obtained transformation frequencies as high as 800 events/g of tissue. However, the batch-to batch variation remains high and we are seeking to optimize it further before adopting it as our standard method.

Despite these improvements, American chestnut remains difficult to establish as somatic cell lines, difficult to transform, and difficult to regenerate into whole plants. Because of these difficulties, a researcher attempting this protocol should expect to spend 6 mo to 1 yr preparing suitable explants, approx 6 mo transforming, recovering, and testing transformation events, and an additional yr regenerating whole plants from the transformed cell lines.

This protocol assumes that the user is familiar with somatic embryogenesis, has experience in *Agrobacterium*-mediated transformation with a model species such as petunia or hybrid poplar, and has a suitable *Agrobacterium* strain containing a plasmid construct with scorable and selectable marker genes flanking one or more genes of interest. For this protocol, we assume the experimenter will be using a *bar* gene as a selectable marker and a *gfp* gene as a scorable marker.

2. Materials

2.1. Specialized Equipment and Supplies

1. Green fluorescent protein (GFP) stereoscopic microscope (Nikon SMZ 1500 with P-FLA fluorescence attachment and 2 filter cubes: GFP longpass no. 83458, TRITC no. 83459 or equivalent).

2. Spectrophotometer (equipped for side-arm flasks).
3. Nephelo Flasks (VWR Scientific, Rochester, NY) (*see* **Note 1**).
4. Desiccation plates (60 × 15-mm disposable Petri plates with a single 55-mm sterile filter paper insert, moistened immediately before use with 200 µL of sterile water).

2.2. Solutions and Media

1. Tween-20 solution: 1% (v/v) Tween-20. Make fresh, do not autoclave.
2. Bleach solution: 50% (v/v) household bleach (5–6% sodium hypochlorite) in distilled water, 2 drops Tween-20/100 mL. Make fresh, do not autoclave.
3. Potato Dextrose Agar (PDA + antibiotics): 39 g/L PDA (DIFCO Laboratories, Detroit MI), 50 mg/L rifampicin, and 100 mg/L kanamycin, pH 7.5.
4. *Agrobacterium* (Agro) Growth Medium (Luria–Bertani [LB] broth + antibiotics): 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 50 mg/L rifampicin, and 50 mg/L kanamycin, pH 7.5.
5. Virulence (Vir) induction medium: 2.6 g/L woody plant medium (WPM) (full strength basal salts) (Sigma; cat.no. M6774) 10 g/L sucrose, 10 g/L MES buffer, and 100 µ*M* acetosyringone.
6. Embryo initiation medium (E1): 2.3 g/L WPM salts, 109 mg/L Nitsch & Nitsch vitamins (Duchefa, Haarlem, NL), 1 g/L casein hydrolysate, 1.8 µ*M* 2,4-D, 1.1 µ*M* 6-benzylaminopurine (BA), 30 g/L sucrose, pH. 5.5, and 3 g/L Phytagel.
7. *Agrobacterium* kill (Agro Kill) medium: E1 with 200 mg/L carbenicillin and 100 mg/L cefotaxime.
8. Selection medium: E1 with 200 mg/L carbenicillin, 100 mg/L cefotaxime, and 1 mg/L glufosinate-ammonium (Sigma) (*see* **Note 2**).
9. Embryo development medium (E2): 2.3 g/L WPM salts, 1 g/L casein hydrolysate; 0.5 g/L L-glutamine (Fluka BioChemika cat. no. 49420), 60 g/L sucrose, pH 5.5, and 3.5 g/L Phytagel.
10. Embryo maturation medium (E3): 3.08 g/L Gamborg basal salt mixture (Phytotechnology; cat.no. G768), 0.5 µ*M* 6-benzylaminopurine (BA) (Sigma; cat.no. B-3408), 0.5 µ*M* α-naphthaleneacetic acid (NAA) (Sigma; cat. no. N-0640), 60 g/L sucrose, pH 5.5, and 3.5 g/L Phytagel.
11. Embryo germination medium (E4): 2.3 g/L WPM salts, 500 mg/L 2-(*N*-morpholino)ethanesulfonic Acid (MES) (Phytotechonogy; cat.no. M825), 500 mg/L polyvinylpyrrolidone (PVP) (Sigma; cat. no. PVP-40), 30 g/L sucrose, pH 5.5, and 3.5 g/L Phytagel.
12. Chestnut multiplication medium (CHN II): 2.3 g/L WPM salts, 109 mg/L Nitsch & Nitsch vitamins; 10 µ*M* Fe-EDTA; 6 m*M* $CaCl_2$, 3 m*M* $MgSO_4$, 1 µ*M* BA; 0.5 µ*M* indole butyric acid (IBA), 35 g/L sucrose, pH 4.5, and 3.5 g/L Phytagel (*see* **Note 3**).
13. Pre-rooting medium (PR low BA): 2.3 g/L WPM salts, 109 mg/L Nitsch & Nitsch vitamins, 500 mg/L MES, 500 mg/L PVP-40, 0.22 µ*M* BA, 30 g/L sucrose, pH 5.5, and 3.5 g/L Phytagel.
14. Rooting medium: 2.15 g/L Murashige and Skoog (MS) salts (Sigma), 30 g/L sucrose, 2 g/L activated charcoal (Sigma; cat.no. C 6289), pH 5.5, and 3.5 g/L Phytagel. (*see* **Note 4**).

2.3. Plant Material

Immature burs of American chestnut collected approximately one month post-anthesis (*see* **Note 5**).

2.4. Agrobacterium *Strains and Vectors*

Agrobacterium strain EHA 105 (*25*) carrying binary vector, pVspB-OxO (*26*) was used for all transformation studies (*see* **Note 6**).

3. Methods

3.1. Somatic Embryo Establishment

1. Collect immature burs approx 1 mo post-anthesis (*see* **Note 5**).
2. Wearing gloves and using a scalpel, remove the spines from the burs.
3. Cut a slice of bur tissue from the top to expose the seed chamber.
4. Using a combination of scalpel cuts and hand pealing, remove the sides of the bur until the nuts (usually 3/bur) are fully exposed. Then break or cut them loose from the base of the bur and place 15 to 20 nuts in a 100-mL wide-mouth bottle with a screw cap (*see* **Note 7**).
5. Cover the nuts with 70% ethanol, cap the bottle, and shake for 20 s. Decant the alcohol into a waste beaker (*see* **Note 8**).
6. Pour enough of the 1% Tween-20 solution to cover the nuts, recap the bottle and shake vigorously for a few seconds. Shake the bottle every 20 to 30 s for 3 min. Decant the Tween-20 solution.
7. Add sufficient 50% bleach solution to cover the nuts (approx 30–50 mL) and allow them to float freely. Shake vigorously for about 10 s and then every 20 to 30 s for 5 min. Decant the bleach solution.
8. Pour in sterile distilled H_2O and shake for about 10 s, then every 20 to 30 s for 5 min. Decant the H_2O.
9. Repeat the sterile water rinses two additional times for a total of three 5-min rinses.
10. Place one nut in a sterile empty Petri plate.
11. Holding the nut by the pointy end with a sterile fine-point forceps, use a sterile scalpel to cut off the base of the nut about $^1/_4$ to $^1/_3$ of the way up the nut (*see* **Note 9**).
12. Set the nut on the cut end, and then slice vertically down both sides.
13. Holding the nut with the scalpel, use the forceps to peal the nut into two halves.
14. Examine the ovules (*see* **Note 10**).
15. Using the forceps, pin one of the nut halves to the petri plate and using the side of the scalpel as a scoop, transfer the white or cream-colored ovules to Petri plates containing E1 Medium. Incubate in the dark at approx 22 to 23°C.
16. For the first 2 or 3 wk, check the plates every other day for contamination. Rescue the clean ovules from a contaminated plate by moving them to fresh E1 Medium (*see* **Note 11**).
17. After discarding the ovules with fast-growing contaminants, decrease the frequency of checking to once every 7 to 10 d (*see* **Note 12**).
18. After all contaminated ovules have been eliminated, decrease the transfer frequency to once every 2 to 3 wk.

19. Usually after 4 to 6 wk (2 or 3 transfers), enough tissue will have emerged from the ovules to require subdividing. For the first few subculture cycles, keep all healthy tissues. Place all of the clumps from one ovule in a single E1 Petri plate (*see* **Note 13**).
20. After 2 or 3 subculture cycles there should be 10 to 12 pieces of tissue from each cell line to choose from. Discard all cell lines that are producing only nonembryogenic callus. Even in the cell lines that are producing somatic embryos many of the clumps will be callus. Discard these clumps. Many of the remaining clumps will contain both callus and embryos, gently cut off and transfer only the somatic embryo clumps, discarding the callus.
21. After three or four more subculture cycles, the remaining cell lines will have developed identifiable characteristics. Some will produce over 90% callus in each clump with very few new embryos. Discard these cell lines—they won't get any better. Some cell lines will produce mostly embryos with 50% or less of each clump becoming callus. Keep subculturing these lines, retaining only the best embryos from the best clumps from each cycle. A small fraction of the cell lines will produce clumps that appear to be all embryos. These cell lines are ready to multiply up for transformation (*see* **Notes 14** and **15**).

3.2. Agrobacterium *Inoculum Preparation*

1. Streak *Agrobacterium* cultures (from a previous plate or from –80°C freezer stocks) onto a fresh Petri plate of PDA + antibiotics.
2. Incubate for 18 to 72 h in the dark at 28°C (*see* **Note 16**).
3. Prepare fresh Agro growth medium and dispense 50 mL into each of 2 side-arm flasks (*see* **Note 17**).
4. Inoculate one side-arm flask with *Agrobacterium* (*see* **Note 18**).
5. Incubate at 28°C on a shaker at approx 200 rpm in the dark (*see* **Note 19**).
6. After the flask containing the *Agrobacterium* begins to look cloudy, start taking hourly OD_{650} readings (*see* **Note 20**).
7. When the OD_{650} reading is between 0.8 and 1.0, transfer the *Agrobacterium* to 2 sterile 50-mL centrifuge tubes (approx 25 mL each).
8. Centrifuge at 1700*g* for 15 min.
9. Pour out the supernatant.
10. Resuspend each pellet in approximately 25 mL Vir induction medium and vortex until the pellet disappears.
11. Transfer both samples to one sterile empty side-arm flask.
12. Incubate at 20–22°C on the shaker (approx 75 rpm—just enough to form a wave) for 4 h (*see* **Note 21**).
13. The *Agrobacterium* inoculum is now ready to use.

3.3. Somatic Embryo Transformation

1. Starting with vigorously growing embryo clumps 1 to 2 wk since their last subculture, transfer somatic embryo clumps to a sterile empty Petri plate (*see* **Notes 15** and **22**).
2. Add enough *Agrobacterium* inoculum to the plate to cover the clumps (10–20 mL). Make sure the clumps are completely wet with inoculum.

3. Incubate in the hood for 1 h.
4. Remove the inoculum with a pipettor or transfer pipet until the embryos are mostly dry.
5. Transfer the embryo clumps to desiccation plates. They should be placed in 5-mm diameter piles with approx 1 cm between the piles.
6. Incubate in the dark at room temperature for 2–3 d (*see* **Note 23**).
7. Transfer the embryo clumps to Petri plates containing Agro kill medium. Clumps should be spread over the surface in a thin layer so that each clump makes contact with the medium. Incubate in the dark at room temperature.
8. After 1 wk, check for GFP expression and transfer all of the embryo clumps to Petri plates containing selection medium (*see* **Note 24**).
9. Transfer and subculture clumps every 2 wk to fresh selection medium (*see* **Note 25**).
10. Six wk after the first transfer to selection medium, start transferring only clumps that show GFP expression (*see* **Note 26**).
11. Multiply each transformation event on selection medium, transferring to fresh medium every 2 to 3 wk, until there are at least 100 clumps of somatic embryos for each transformation event.
12. Screen each GFP-positive putative transformation event for the presence of at least one, and preferably each, of the genes of interest (*see* **Note 27**).

3.4. Conversion of Somatic Embryos Into Multiplying Shoot Cultures

1. Starting with healthy, vigorously growing (a maximum of 3 wk since the last transfer), and uniformly GFP-positive chestnut somatic embryo cultures; transfer clumps onto Petri plates containing E2 medium.
2. Incubate the embryo clumps in the dark at 23–25°C for 4 wk.
3. Using a stereomicroscope, excise and transfer individual cotyledonary stage embryos to Petri plates containing E3 Medium (*see* **Note 28**).
4. Incubate the embryos in the light (40 μmol/m^2/s, 16 h/d) for 7 d.
5. Transfer the embryos to a Magenta GA-7 vessel containing E4 Medium.
6. Incubate in the light (40 μmol/m^2/s, 16 h/d).
7. Transfer the embryos to fresh E4 Medium every 2 wk (*see* **Note 29**).
8. Watch the embryo masses for new shoot formation (*see* **Note 30**).
9. Transfer shoots to CHN II medium as they appear.
10. Multiply the transgenic shoots on CHN II medium until there are at least 100 clumps of shoots of each transformation event (*see* **Note 31**).

3.5. Rooting and Acclimatization of Chestnut Microshoots

1. Starting with healthy and vigorously growing chestnut shoot cultures (a maximum of 4 wk since the last transfer), select shoots greater than 2 cm in length.
2. Excise and discard the shoot tip. Place the shoot in fresh CHNII Medium in a Magenta GA-7 vessel, with approx 16–20 shoots/vessel.
3. Incubate shoots on a 16-h photoperiod with a light intensity of 80 μmol/m^2/s at 25°C (*see* **Note 32**).
4. After approx 3 wk, when the new crop of axillary buds are approx 1 to 4 mm in length, transfer them to Magenta GA-7 vessels containing PR low BA medium

and reduce the light intensity to approximately 20 μmol/m^2/s to promote shoot elongation (*see* **Note 33**).

5. Transfer the shoots to fresh PR low BA medium every 2 wk until the majority of the shoots are between 2 and 3 cm in length (*see* **Note 34**).
6. Cut off any callus that may have formed on the base of the elongating shoots. Carefully split the basal end of the shoots between 1 and 2 mm up the stem (*see* **Note 35**).
7. Dip the cut end in a freshly prepared, filter sterilized solution of 10 m*M* IBA for 60 s. (approx 10 mL of the IBA solution in a 60-mm Petri plate will provide the appropriate depth to submerge just the end of the shoots. Getting the IBA on the stem higher than this will cause callus to form) (*see* **Note 36**).
8. Immediately after dipping the shoots, transfer them to Magenta GA-7 vessels containing rooting medium and culture for 10 to 12 d under 40 μmol/m^2/s 16-h/d (*see* **Note 37**).
9. Transfer shoots to PR low BA medium and culture for 1 mo (*see* **Note 38**).
10. Prepare a soilless potting mixture in the combination of 2:1:1 peat:perlite:vermiculite. Moisten this with a water-soluble fertilizer solution (*see* **Note 39**).
11. Thoroughly clean enough 4-in. square plastic pots and Magenta GA-7 vessels (for lids) so there is one/plantlet (*see* **Note 40**).
12. Rinse the pots and Magenta vessels in a 1% bleach solution followed by several clean water rinses. Water collecting trays should also be as clean as possible.
13. Fill the pots with the potting mixture to within 2 cm of the top.
14. Rooted plantlets should be removed carefully from the gelled medium. Take care to wash the roots free of all gelled medium (*see* **Note 41**). Remove one plantlet at a time to reduce transpiration stress.
15. Place one plant per pot, tamp down the potting mix around the roots, and invert a Magenta GA-7 vessel over the plantlet. Press the vessel down slightly so it is in good contact with the potting mix.
16. Water the plantlet with 50 mL of room temperature tap water.
17. Place the plants in a controlled environment with a 16-h day length at 90 to 150 μmol/m^2/s light intensity and 20°C day temperature. Ambient relative humidity should be maintained at 65% (*see* **Note 42**).
18. Monitor the plants daily for signs of desiccation during the first wk. If plants or potting mix look dry, water with room-temperature tap water.
19. After 1 wk, begin acclimatizing the plantlets to ambient humidity by gradually lifting the inverted Magenta GA-7 vessel. Raise one side of the vessel and prop it up on the side of the pot so that air can freely exchange through one side and partially through two other sides. At this stage, plants should be monitored twice daily for signs of wilting and for potting mix moisture. If wilting occurs, close the lids until plants regain turgidity,
20. Plants should be watered approx twice a wk with 100 mL of tap water.
21. One wk after the lid has been raised, remove it entirely. Monitor the plants for wilting.
22. After another 4 wk of exposure to ambient relative humidity in the growth chamber, the acclimatized plantlets can be moved to a shaded greenhouse (*see* **Note 43**).

4. Notes

1. Nephelo side-arm flasks can be inserted directly into a spectrophotometer equipped with a suitable adapter, allowing for a quick nondestructive determination of optical density.
2. We usually substitute the herbicide Finale®, which contains the active ingredient phosphinothricin (PPT) for reagent grade glufosinate-ammonium.
3. The extra calcium, magnesium, and iron are added on the basis of personal correspondence with Ms. Malinie Barker, USDA Forest Service, North Central Forest Exp. Station, St. Paul, MN.
4. The charcoal should be dispensed into empty individual containers and autoclaved separately. The autoclaved and partially cooled (approx 55°C) rooting medium should then be added to the containers in a laminar flow hood, swirled briefly to mix, and allowed to cool.
5. Collect only from chestnut trees in flowering groups. *Castanea* is almost completely self-sterile so ovules from isolated trees will abort. Expect to see large tree-to-tree differences in contamination rates and ease of establishment of cell lines, so it is better to collect a few burs (perhaps 10 to 15) from many different trees rather than a lot of burs from a few trees. Burs can be kept at 4°C in sealed plastic bags for 1 wk or longer, but if the spines turn brown, the ovules inside are probably brown too.
6. The pVspB-OxO vector carries three genes: A selectable marker (*bar*), a scorable marker (*gfp*), and a putative blight-resistance gene (*OxO*). The *bar* gene codes for a phosphinothricin acetyl transferase ***(27)***. It carries a potato ubiquitin (Ubi3) promoter and terminator ***(28)***. The *gfp* gene codes for a modified green fluorescent protein (*mgfp5-ER*) ***(29)***. It carries a CaMV 35S promoter and terminator. The *OxO* gene codes for a wheat germin-like oxalate oxidase gene ***(30)***. It is driven by a soybean vegetative storage protein (VspB4) promoter ***(31)*** and has an Actin 2 (Act2) terminator. Details of the construction of pVspB-OxO are in **ref.** ***26***.
7. Be careful not to cut into the nut or else the bleach solution will kill the ovules. Treat only as many nuts as can be processed in 1 d. With practice, it is possible to extract and plate out ovules from about 15 to 20 nuts in 1 h, but it is tiring because of the tough seed coats. We found that one person could work for about 3 h at a sitting.
8. **Steps** 5–43 on should be conducted in a laminar flow hood.
9. In immature chestnuts, all the ovules are in a little cluster at the pointy end of the nut.
10. *Castanea* nuts are polyembryonic with 12 or more ovules in each nut. Soon after fertilization all the ovules begin to grow, but within 6 wk, one will have expanded to fill the nut while the rest will have aborted. The optimum stage of development for establishing somatic embryo cell lines is when the cluster of ovules has begun to develop but the dominant ovule is still less than three times the size of the other ovules in the group. If the largest ovule in the cluster is too big and the others are brown or black, discard the nut.
11. Expect a high contamination rate. Also expect some trees to have higher contamination than others.

12. There is usually a burst of initial contamination from fast-growing fungi and bacteria, but slow-growing bacteria can show up after 1 mo or more. If apparently "clean" ovules are repeatedly "rescued", these slow-growing contaminants may not be discovered for many wk.
13. A broad range of tissue types will emerge from the ovules. The key is being able to distinguish between callus and somatic embryos. Somatic embryos look like tiny clusters of balloons or grapes. They are smooth and regular in shape. Callus is more rough surfaced and irregular in shape and usually harder than the somatic embryos.
14. It requires a large number of nuts, preferably collected from a number of different trees, to eventually end up with a small number of vigorous somatic cell lines. In 2004, our lab received burs from 18 trees, explanted more than 3000 ovules, and 6 mo later we had 5 cell lines suitable for transformation studies.
15. If cell cultures have been stressed by infrequent transfers to fresh medium, transformation efficiency will plummet. If possible, use cell lines that multiply as early-stage proembryogenic masses.
16. Store the plates at 4°C if they will be needed after 72 h. Make a new plate if previous one is over 1- wk old.
17. Add the filter sterilized antibiotics (after autoclaving the LB broth and cooling to room temperature) to both the flask that will be inoculated with *Agrobacterium* and the one that will be used as a blank to zero the spectrophotometer. Rifampicin changes the OD reading.
18. Some protocols use one or more overnight cultures in small volumes of medium. We initiate our main batch of inoculum directly from a fresh streak plate with a large loopful of *Agrobacterium*, but then watch the OD_{650} reading closely.
19. *Agrobacterium* will grow fine in the light; the antibiotics, however, are light-sensitive.
20. Initiated from a fresh plate, the *Agrobacterium* will begin to cloud the medium after as little as 12 h. When using an old plate, it may take 24 h or longer.
21. The purpose of this step is to induce the *vir* genes, not to grow more bacteria.
22. Some clones produce masses of tiny friable clumps of cells that are extremely tedious to transfer. These can be treated in place by flooding the embryos with *Agrobacterium* inoculum without transferring them to an empty Petri dish. Remove the excess inoculum after 15 min, co-cultivate for 2 to 3 d, and then transfer embryos to selection medium (skipping the desiccation and the Agro kill treatments).
23. Chestnut somatic embryo cultures should be maintained in the dark at room temperature in this and subsequent stages. The creamy white color makes GFP expression easy to see.
24. GFP expression can usually be observed after 1 wk. If everything went well, there should be dozens of tiny GFP-positive foci/clump of embryos. It is best, however, to transfer all the embryo clumps rather than visually select GFP-positive cells at this time.
25. Many things are going on simultaneously, any remaining *Agrobacterium* cells are dying, nontransformed plant cells are dying, many of the GFP-positive events are failing to divide (transient events), some of the transformed cells are starting to

divide and form fluorescing sectors of cells, and some of these sectors are forming callus while others are forming tiny new clumps of embryos.

26. We find it most efficient to transfer everything for the first two or three subcultures and then look among the survivors for sectors that are still embryogenic and have high GFP expression. Immediately before each transfer, check for bacteria colonies growing out from the embryo clumps. Sometimes a few *Agrobacterium* cells will survive for several months. Discard any contaminated embryos and go back to a 2-wk transfer cycle.
27. Screening transformation events for correct DNA integration is an important follow up for the transformation process, but requires equipment and expertise well beyond that available in most tissue culture laboratories. The simplest screen is to use PCR; this technique, however, is prone to false positives and cannot be used to determine how many copies of the genes of interest have been incorporated into a transformation event. Southern hybridization assays require considerably more tissue for extracting DNA but are more reliable and can be used to determine copy number.
28. Many of the embryos may be abnormal (normal embryos will have 2 cotyledons). They may have only one cotyledon, the cotyledons may be fused, or there may be more than two cotyledons. Transfer all of them, as this does not seem to affect the subsequent regeneration process.
29. The embryos will grow into unrecognizable lumpy green masses, 1 to 2 cm in size. Don't be overly concerned. Some will eventually form morphologically-normal shoots.
30. Shoots should begin to develop 4 to 6 wk after being on E4 Medium.
31. Once a transgenic cell line has been regenerated into a stable shoot culture, it can be maintained for years by subculturing every 4 to 6 wk onto fresh CHN-II medium in Magenta vessels.
32. This is approx twice the light intensity we use for standard multiplication stage cultures. Lower light levels promote shoot elongation and at this stage we are trying to promote axillary bud development.
33. Many of the new buds will appear pink in color. Select these buds because they will grow rapidly.
34. This usually takes 2 or 3 transfers. Shoots larger than 3 cm root poorly, but shoots as small as 1 cm may be used if necessary.
35. Use extreme caution and a very sharp scalpel. Make sure you are slicing through the stem and not crushing the tissue.
36. To hasten this procedure, you can dip a batch of shoots together by making a stand for them out of the perforated rack from a pipet tip box cut to approx 3×4 cm by 1-cm high. After each shoot is sliced, place it in the stand until you have 10 shoots at a maximum. Keep the shoots moist by placing the stand in a sterile Petri plate with approx 5-mm of sterile water. Once ten shoots have been prepared, transfer the whole stand to a Petri plate containing the IBA solution and proceed as above.
37. During this stage, it is normal to witness shoot-tip die back. In some cases it can be as severe as 50% of the total shoot height.

38. Roots may be visible during the 10 to 12 d in the rooting medium but transfer all shoots, even those not rooted, to the PR low BA. More shoots will produce roots during this culture cycle. Shoots that are not rooted at the end of this cycle may be discarded. This stage will also allow for an axillary bud to elongate.
39. The fertilizer solution should contain the full compliment of macro and micronutrients and should be prepared at the lowest concentration recommended. Do not autoclave this mixture. Estimate 500 mL of potting mixture per plantlet. Depending on the quality of peat, you may need to break up large clumps by hand. It is easier to mix the dry components and then add the nutrient solution.
40. Even though the soil mix is not sterile it is important to maintain a high degree of cleanliness in this step.
41. The roots will be very fragile. It is helpful to break up the solid medium with forceps before removing the plantlets. This stage is the beginning of nonsterile conditions, which is why all of the tissue culture medium must be removed from the roots. Any medium left on the roots will promote fungal growth. As an additional precaution, plantlets can be dipped in a fungicide solution.
42. It is important to begin increasing the light level and varying the temperature at this stage.
43. For the combined rooting and acclimatization process, expect to see an overall survival rate of 10 to 20%. In the greenhouse and subsequent field planting, we have found that micropropagated plantlets lag behind seedlings of the same age for the first growing season.

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23

Apple (*Malus × domestica*)

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Summary

Apple (*Malus × domestica*) is one of the most consumed fruit crops in the world. The major production areas are the temperate regions, however, because of its excellent storage capacity it is transported to distant markets covering the four corners of the earth. Transformation is a key to sustaining this demand—permitting the potential enhancement of existing cultivars as well as to investigate the development of new cultivars resistant to pest, disease, and storage problems that occur in the major production areas. In this paper we describe an efficient *Agrobacterium tumefaciens*-mediated transformation protocol that utilizes leaf tissues from in vitro grown plants. Shoot regeneration is selected with kanamycin using the selectable kanamycin phosphotransferase (APH(3)II) gene and the resulting transformants confirmed using the scorable *uidA* gene encoding the bacterial β-glucuronidase (GUS) enzyme via histochemical staining. Transformed shoots are propagated, rooted to create transgenic plants that are then introduced into soil, acclimatized and transferred to the greenhouse from where they are taken out into the orchard for field-testing.

Key Words: Apple; plant transformation; *Agrobacterium tumefaciens*; regeneration; propagation; transgenic plants; fruit.

1. Introduction

Agrobacterium-mediated transformation remains the most successful method to introduce novel genes into plants ***(1)***. Within the Malus genera, apple (*Malus × domestica*) is the species that best provides a reproducible system for transformation. This has been made possible by the formulation of a high-efficiency, reproducible, in vitro regeneration system ***(2–5)***. Transgenic apple production has been the result of transformation experiments with disarmed strains of *Agrobacterium* containing the binary plasmid vectors pBIN6 ***(6)*** and pCGN ***(7)*** incorporated via electroporation ***(8)***. One of the apple cultivars that perform best is 'Greensleeves' because it grows and roots vigorously in vitro, and is less prone

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to vitrification *(9)*. Axillary shoots of the 'Greensleeves' cultivar can be maintained in vitro by transfer to fresh media at 1-mo intervals. One of the limitations of the apple tissue culture has been that some cultivars have proven to be difficult to root in vitro. For this reason a two-phase method has been developed, which includes a 2- to 4- d induction phase on an auxin-containing media and an emergence phase in a hormone-free media with half strength Murashige and Skoog (MS) microelements *(10)*. Although a variety of tissues can be used for transformation, leaves have become the organ of choice because of their abundance and ease of manipulation for infection with *A. tumefaciens*. Leaf explants are obtained with the use of a cork borer *(11)*. The leaf explants offer some advantages compared to other segments as they behave more consistently and the tissue grows such that there is better contact with the selected media. Leaf explants are infected with *A. tumefaciens*, which have been grown overnight and resuspended in virulence induction media for 5 h and co-cultivated for 3 d *(12)*. After co-cultivation, explants are transferred to regeneration media and shoots generally appear within 2 to 9 mo, depending on the concentration of kanamycin. The transformation efficiency achieved with this method is 1.5 GUS positive shoots for every 100 leaf explants. To describe the transformation procedures we will begin with establishment, propagation, and rooting of the cultivar 'Greensleeves' and continue through to the infection with *A. tumefaciens*, regeneration and finally the selection of transgenic apple trees.

2. Materials

2.1. Plant Material

Leaf explants from previously established in vitro growing apple shoots obtained from the apple scion variety 'Greensleeves'.

2.2. Transformation Vector and A. tumefaciens Strain

1. *Agrobacterium tumefaciens* strains that work efficiently for apple are the disarmed derivatives like EHA101 *(13)* of the tumorigenic A281 strain that harbors the Ti plasmid pTIBo542 and the nonpathogenic strain C58C1 *(14)* that contains a disarmed version of the tumorigenic Ti plasmid pTiC58.
2. The binary system for apple transformation is completed with the introduction of broad host range binary plasmids that contained the desired T-DNA region, for this we use derivatives of binary plasmids described by McBride and Summerfelt *(7)*. These derivatives have been exclusively used for the *Agrobacterium*-mediated transformation protocol described here. This binary contains the selectable marker gene APH(3)II for kanamycin resistance and has been modified to contain the scorable marker gene *uidA* encoding GUS. Binary vectors can be introduced into *Agrobacterium* strains by a number of methods as described in Chapter 3, Volume 1 (*see* **Note 1**).

2.3. Stock Solutions

1. 1000X MS vitamin stock: 0.5 g/L thiamine HCl, 0.5 g/L pyridoxine HCl, 0.05 g/L nicotinic acid, and 2.0 g/L glycine. Store at 4°C up to 4 mo.
2. Indole-3-butyric acid (IBA): 1 mg/mL stock solution. Dissolve 10 mg of powder in 0.3 mL of 1.0 *M* KOH. Add deionized water and filter sterilize. Store at 4°C for up to 1 mo.
3. 6-Benzylaminopurine (BAP): 1 mg/mL stock solution. Dissolve 10 mg of powder in 0.3 mL of 1.0 *M* KOH. Add deionized water and filter sterilize. Store at 4°C up to 1 mo.
4. α-naphthaleneacetic acid (NAA): 1 mg/mL stock solution. Dissolve 10 mg of powder in 0.3 mL of 1.0 *M* KOH. Add deionized water and filter sterilize. Store at 4°C up to 1 mo.
5. Thiodiazirunon (TDZ): 0.1 mg/mL stock solution. Dissolve 10 mg of powder in 0.3 mL of 1.0 *M* KOH. Add deionized water and filter sterilize. Store at 4°C up to 1 mo.
6. *myo*-Inositol: 1 mg/mL stock solution. Dissolve powder in water and filter sterilize it. Store at room temperature up to 3 mo.

2.4. Media

1. Shoot multiplication media (SM/A17): 30 g/L of sorbitol, 4.31 g/L of MS macroelements, 1 mL/L of *myo*-inositol (1 mg/mL stock solution), 1X MS vitamin, 1 mL/L of BAP (1 mg/mL stock solution), 0.1 mL/L IBA (1 mg/mL stock solution), pH 5.8, with 1.0 *M* potassium hydroxide (KOH), add 8 g/L Bacto agar. Autoclave for 20 min (*see* **Note 2**).
2. Root induction media (RI/ R13): 30 g/L of sorbitol, 4.31 g/L of MS macroelements, 1 mL/L of *myo*-inositol (1 mg/mL stock solution), 1X MS vitamin, 3 mL/L IBA (1 mg/mL stock solution). Adjust the pH to 5.8 with 1.0 *M* KOH. Add 8 g/L Bacto agar and autoclave for 20 min.
3. Root emergence media (RE/ R37): 30 g/L of sorbitol, 2.15 g/L of MS macroelements, 0.5 mL/L of myo-inositol (1 mg/mL stock solution), 0.5X MS vitamin. Adjust the pH to 5.8 with 1.0 *M* KOH. Add 8 g/L Bacto agar and autoclave for 20 min.
4. Co-cultivation media (CC): 30 g/L of sorbitol, 4.31 g/L of MS macroelements, 1 mL/L of *myo*-inositol (1 mg/mL stock solution), 1X MS vitamin, 5 mL/L BA (1 mg/mL stock solution), 1 mL NAA (1 mg/L stock solution), 1 mL/L TDZ (1 mg/L stock solution). Adjust the pH to 5.8 with 1.0 *M* KOH. Add 8 g/L Bacto agar and autoclave for 20 min. Cool down in water bath at 52°C, add 1 mg/L proline (100 mg/mL stock solution and 100 μ*M*/L acetosyringone (200 m*M* stock solution).
5. Regeneration media (RG): 30 g/L of sorbitol, 4.31 g/L of MS macroelements, 1 mL/L of *myo*-inositol (1 mg/mL stock solution), 1X MS vitamin, 5 mL/L BA (1 mg/mL stock solution), 1 mL NAA (1 mg/L stock solution), 1 mL/L TDZ (1 mg/L stock solution). Adjust the pH to 5.8 with 1.0 *M* KOH. Add 8 g/L Bacto

agar and autoclave for 20 min. Cool down in water bath at 52°C add 200 μg/mL cefotaxime and 100 μg/mL kanamycin.

6. *Agrobacterium* growth medium (YEP): 5 g/L Bacto yeast extract, 10 g/L of Bacto peptone, and 10 g/L of NaCl; adjust pH to 7.2. Add Bacto-agar (15 g/L) to prepare solid YEP medium.
7. Virulence induction medium (IM): 1X MS salts, 1X MS vitamins, 2% sucrose, 100 mg/L *myo*-inositol, 1 m*M* proline, 100 μM acetosyringone. Adjust the pH to 5.2 and filter sterilize (do not autoclave). Store at 4°C up to 1 d, if needed.

2.5. Other Chemicals and Supplies

1. Kanamycin sulfate: 50 mg/mL stock solution. Dissolve the powder in water, and store at −20°C.
2. Gentamycin sulfate: 20 mg/mL stock solution. Dissolve the powder in water, filter-sterilize, and store at −20°C.
3. Cefotaxime, 100 mg/mL stock solution. Dissolve the powder in water, filter-sterilize, and store at −20°C.
4. Acetosyringone: 200 m*M* stock solution. Dissolve the powder in ethanol and store at −20°C.
5. Proline: 100 mg/mL stock solution. Dissolve the powder in water, filter-sterilize and store at 4°C.
6. 5-Bromo-4-chloro-3-indolyl glucuronide (X-Gluc) staining buffer: 100 m*M* sodium phosphate, pH 7.0, 10 m*M* ethylenediamine tetraacetic acid (EDTA), 0.5 m*M* ferrocyanide, 0.5 m*M* ferricyanide, and 0.006% (v/v) Triton X-100. Store at 4°C.
7. X-Gluc 0.02 *M* stock solution: Dissolve the powder in deoxygenated dimethyl formamide (DMF). Store at −20°C.
8. X-Gluc staining solution (10 mL): 9.5 mL X-Gluc staining buffer, 0.5 mL 0.02 *M* X-Gluc stock solution.
9. Bleach solution: 12% (v/v) bleach (0.525% sodium hypochlorite).
10. Potting soil: Any commercial potting soil obtained from Ace Hardware Co.

2.6. Culture Condition

Shoots are multiplied and rooted at approx 25°C under a 16-h photoperiod (fluorescent light, 85 $\mu E/m^2/s$). Co-cultivation is conducted at 21°C in the dark. Regeneration is conducted at approx 25°C in the dark.

3. Methods

3.1. Establishing and Propagating Apple Shoot in Culture

1. Before bud break cut shoots from apple scion and place at room temperature in a beaker with enough water to keep the base wet.
2. Allow auxiliary and terminal buds to elongate to about 3 cm and then cut them with a scalpel (*see* **Note 3**).
3. Wash the shoot tips under running water for 30 min to remove surface contaminants.

4. Sterilize the tips in bleach solution for 20 min (*see* **Note 4**).
5. Transfer all materials to a sterile hood.
6. Rinse the shoot tips with sterile distilled water 6 times at 2-min intervals.
7. Cut 2 to 3 mm of the base of the shoots and any major visible leaves.
8. Place one shoot in each 25-mL vial containing 10 mL SM/A17 (*see* **Note 5**).
9. Every 3 wk, cut 2- cm shoots and transfer to fresh media.
10. Check daily. Should the base produce phenolics and turn brown, cut off 2 to 3 mm of the shoot and transfer it to new SM/A17 medium.
11. Keep shoots derived from a single shoot separated from those derived from other shoots. Make sure to label them properly to keep track of the origin (*see* **Note 6**).
12. Cut terminal and auxiliary shoots and transfer to new SM/A17 medium every 3 to 4 wk until the desired number of shoots is obtained (*see* **Note 7**).

3.2. Root Induction, Emergence, and Explant Preparation

The transformation efficiency increases when the leaf explants are taken from rooted shoots. For this reason it is important to have a constant source of rooted shoots. When the desired number of shoots from SM is achieved, some shoots can be used for rooting (*see* **Note 8**).

1. Cut auxiliary (approx 2 cm) shoots and place them on RI/R13 medium for 3 d at room temperature under a 16-h photoperiod (fluorescent light, 85 $\mu E/m^2/s$) (*see* **Note 9**).
2. Take shoots and without cutting off the base place them on RE. After about 10 d roots should be visible in at least some of the shoots.
3. Three to four wk after shoots are transferred onto RE, the leaf should be of sufficient size to cut a 7-mm disc using a cork borer (*see* **Note 10**).

3.3. Agrobacterium *Culture Preparation*

1. Day 1: Plate *Agrobacterium* from frozen stock onto YEP medium containing appropriate antibiotics and incubate overnight at 28°C (*see* **Note 11**).
2. Day 2: Inoculate 5 mL of YEP with bacteria from plate and incubate with shaking at room temperature for 2 to 3 h.
3. Add appropriate antibiotics to 5 mL of YEP, swirl and combine with *Agrobacterium*-YEP suspension and incubate with shaking overnight at room temperature.
4. Day 3: Determine OD at A_{420} using 100 µL bacteria suspension from overnight growth and 900 µL YEP.
5. Pellet bacterial cells at 5000*g* for 15 minutes and resuspended in IM to 0.5 OD_{420}.
6. Incubate at room temperature with shaking for 5 h.

3.4. Plant Transformation

1. Cut leaf from rooted in vitro plant with a scalpel and float on sterile, distilled water in 9-cm Petri dishes (*see* **Note 12**).

2. Immediately cut leaf discs using a 7-mm cork borer and place in water (*see* **Note 13**).
3. Incubate leaf disc with *Agrobacterium* for 10 to 20 min.
4. Blot leaf disc onto sterile Whatman filter to remove excess bacteria.
5. Transfer to CC. Place 24 discs on each plate.
6. Seal Petri dishes with parafilm. Incubate in the dark at 21°C.
7. Day 6: Transfer to RG.
8. Seal Petri dishes with Parafilm. Incubate in the dark at 25°C. Check weekly for regenerating shoots.
9. Transfer to fresh media at monthly intervals (*see* **Note 14**).
10. Transfer regenerated shoots as soon as they appear to SM/A17 (*see* **Note 15**) supplemented with 200 μg/mL cefotaxime and 100 μg/mL kanamycin (*see* **Note 16**).
11. Incubate under a 16-h photoperiod (fluorescent light, 85 $\mu E/m^2/s$) at room temperature.
12. Subculture shoots at monthly intervals until sufficient material is produced for physiological, biochemical and molecular analyses.

3.5. Histochemical Staining for GUS Activity

When a few shoots from each line are obtained and it is possible to take a couple of pieces of leaf from one of the shoots, it is time to perform a GUS activity assay.

1. Prepare 200 μL X-Gluc staining solution/sample, mixing X-Gluc to X-Gluc staining buffer, and distribute on wells of 96-multi-well plates.
2. Place tissue in wells containing 200 μL of X-Gluc staining solution (*see* **Note 17**).
3. Place the plate at 37°C in the dark overnight (*see* **Note 18**).

3.6. Rooting of Transgenic Shoots and Transplanting to Soil

A two-phase method is also used in this case: root induction and root emergence.

1. Subculture shoots in RI supplemented with 200 μg/mL cefotaxime and 100 μg/mL kanamycin. Place them under a fluorescent light (85 $\mu E/m^2/s$) with a 16-h photoperiod for 3 d (*see* **Note 19**).
2. Take shoots (without cutting off the base) and place them on RE supplemented with 200 μg/mL cefotaxime and 100 μg/mL kanamycin under a 16-h photoperiod (fluorescent light) (*see* **Note 20**).
3. After the root induction, let plant grow in RE medium for about 1 mo (*see* **Note 21**).
4. Place some potting soil in a seedling tray (*see* **Note 21**) to cover the bottom.
5. Hold the plant in place and add the rest of the soil, which should cover up the first leaves, and press gently.
6. Water the plant while keeping it as straight as possible.
7. Cover the plant with a clear plastic cup and transfer it to the green house (*see* **Note 22**).
8. After a few days start punching holes in the cup (one hole/d) (*see* **Note 23**).
9. Let plant grow for several more days before transplanting to a bigger pot (*see* **Note 24**).

10. Transgenic lines are maintained in the greenhouse/lathouse and in the field as scions. Our greenhouse conditions are: 25°C, 16 to 18 h photo period (25°C d and 20°C night), 800 to 1000 μE/m^2/s light intensity at 3 ft from ground level.

4. Notes

1. We use electroporation to introduce DNA intro *Agrobacterium*. Briefly, 1 μL of plasmid DNA (10–100 ng) is added to *Agrobacterium* competent cells. The mixture is placed on ice for 2 min before being transferred to a pre-cooled 0.2-cm electroporation cuvet (BioRad). Apply voltage with settings at 2.5 kv (field strength), 25 μF (capacitance), 400 Ω (resistance) at time constants of 8 to 12 m/s. One milliliter of YEP rich media is added to the electroporated cell/DNA mixture. Incubate it with shaking at room temperature for 45 min and then plate on selective media.
2. Swirl the media thoroughly before pouring it into petri dishes (9 cm). When antibiotics need to be added, this should be done once the media cools down in the water bath of 55°C as they are unstable at high temperatures. Swirl the media thoroughly after the addition of the antibiotics.
3. Apical buds reach an adequate size within 5 d while lateral buds take slightly longer. Shoot tips should consist of the apex plus $^2/_3$ axillary buds, or alternatively, cut off terminal buds and the axillary buds will break. Store some shoots at 4°C up to 3 to 4 mo for later use in the case of contamination of the first set put into culture.
4. Usually about 10 shoots will fit in a 50-mL falcon tube containing about 45 mL of solution, which can then be set on a rocking platform. It is important not to overcrowd the tube so that shoots can move freely.
5. It is essential at this point to use small vials containing a single shoot because most of the shoots are still contaminated after the sterilization in bleach. After shoots are established they can be propagated in small single shoot vials, magenta boxes, or deli cups. There are pros and cons for each of those. In Dilu vials, place only one shoot to avoid cross contamination. Deli cups can be a big timesaver, as it takes less time to pour the media and transfer the shoots and, unlike magenta boxes, are disposable. For shoot multiplication, we use 10-cm diameter, clear deli cups with 100 mL SM/A17. In those cups we place between 7 and 9 shoots.
6. After 20 to 30 shoots are obtained from a single bud choose among these and keep the one that is propagating the best and free of contamination. All the transformations are performed from a single line of shoots.
7. Usually the apical 2 cm of each terminal and axillary shoot is used, but to speed up the multiplication process other segments below can be used. Ensure 10 to 15 mL of media for each shoot. Shoot growth and multiplication is slow in the first 2 to 3 mo. It usually takes more than 6 to 8 mo to obtain a sufficient number of shoots to use for a large-scale experiment.
8. Shoot multiplication is the crucial step, and it is important to root each time what can be used for transformation in 1 or 2 d. This should be only part of the propagated shoots to guarantee a constant source of freshly propagated material.

9. Shoots can be placed at high density on RI because they stay in the same media only for 3 d. Depending on the size, 50 to 100 shoots can be placed in one deli cup containing 100 mL of RI.
10. Great care must be taken in sharpening the cork borer. This might take some time. The way leaf discs are cut is also important. Press firmly on the leaf and rotate the weight applied around the blade without twisting the blade. The hand/handle should move in circle around the axis perpendicular to leaf/table surface.
11. The plate can be stored at 4°C for a few mo and used for inoculation. Seal plate with parafilm.
12. Leaves are placed in water with the adaxial (upper) face up. For very small experiments, and with much practice, it can be done directly in IM.
13. Because it takes sometime to cut discs for large experiments, start to prepare explants soon after bacterial cells are resuspend in IM media. Use the minimum amount of water necessary to cover the surface of the Petri dishes used to store and infect the discs with agro. Make sure the whole disc is touching the water and that the adaxial face is up.
14. Intervals can be shorter depending on the growth of the discs, the formation of condensation and the use of transpiring film instead of parafilm.
15. It is useful at this point to keep single shoots in small Dilu vials to avoid cross contamination of the precious material. Dilu vials are loaded with about 10 mL of SM/A17.
16. Shoot may appear within 2 to 9 mo, usually from the mid-rib. When removing the disc or explant with regenerated shoot from the RG media, handle only the leaf disc or explant, not the shoot to avoid damaging the new shoot.
17. Antibiotics selection is not enough to guarantee that all regenerated shoots are transgenic and escapes are usually present.
18. Usually the blue color is visible after a few hours, but the best result is obtained after overnight staining.
19. Rooting in media containing kanamycin is important for two reasons. First, it is a further warranty of the presence of the insert in the plant, and second, to make sure that the shoot can generate a whole plant.
20. After about 10 d, roots should be visible at least in some shoots.
21. Before transplanting, the root should be very strong. Use a black seedling tray (25 × 50 cm) that holds 24 plants.
22. This should be big enough to contain the whole canopy of the plant for one-month growth.
23. After 1 wk or more try to leave a few plants without the cup for 1 h, checking often to be sure that they are doing well and do not wilt.
24. Plants can now be kept in the greenhouse or transferred to the field, depending on the season.

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Blueberry (*Vaccinium corymbosum* L.)

Guo-Qing Song and Kenneth C. Sink

Summary

Recent advances in plant biotechnology have led to a reliable and reproductive method for genetic transformation of blueberry. These efforts built on previous attempts at transient and stable transformation of blueberry that demonstrated the potential of *Agrobacterium tumefaciens*-mediated transformation, and as well, the difficulties of selecting and regenerating transgenic plants. As a prerequisite for successful stable transformation, efficient regeneration systems were required despite many reports on factors controlling shoot regeneration from leaf explants. The *A. tumefaciens*-mediated transformation protocol described in this chapter is based on combining efficient regeneration methods and the results of *A. tumefaciens*-mediated transient transformation studies to optimize selected parameters for gene transfer. The protocol has led to successful regeneration of transgenic plants of four commercially important highbush blueberry cultivars.

Key Words: GUS; regeneration; transformation; transgenic plant; transient expression; woody plant.

1. Introduction

Blueberries (*Vaccinium* sp.) have a long history of use as food and medicine in Europe and North America. They are generally regarded as one of the richest sources of antioxidant phytonutrients of the fresh fruits and vegetables that have been studied *(1,2)*. Traditional breeding approaches for blueberry are time consuming and labor-intensive because of its heterozygosity, polyploidy, and length of evaluation trials. Transformation is particularly suited to blueberry because it has a polyploid genome and is asexually propagated.

Adventitious shoot regeneration systems from leaf explants of highbush blueberry have been developed for several cultivars *(3–7)*, and, although used in transient expression studies, the methods did not lead to a reliable transformation method. There have been two reports of *Agrobacterium tumefaciens*-mediated

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transient or stable transformation of blueberry *(8,9)* as well. *A. tumefaciens*-mediated transformation of the hybrid blueberry cv. North Country (*Vaccinium corymbosum* × *V. angustifolium*) was reported, but the transformants, which were obtained without an antibiotic-selection method because of the extreme sensitivity of the leaf explants to kanamycin, were not confirmed by Southern analysis *(8)*. Cao et al. studied several factors influencing transient β-glucuronidase (GUS) expression, including sucrose concentration in the medium *(10)*, time of co-cultivation, *A. tumefaciens* strain, explant age, and genotype *(9)*. Four d of co-cultivation with EHA105 yielded efficient transient GUS expression, and genotype and explant age were also found to be important factors.

We recently established a protocol for transformation of highbush blueberry (*Vaccinium corymbosum* L.) cultivars by *A. tumefaciens* *(11)*. Leaf explants of the cultivars Aurora, Bluecrop, Brigitta, and Legacy are inoculated with strain EHA105 containing the binary vector pBISN1 with the neomycin phosphotransferase gene (*npt* II) and an intron interrupted GUS reporter gene (*gusA*) *(12–14)*. Co-cultivation is for 6 d on modified McCown's woody plant medium (MWPM2S) *(15)* containing 100 μ*M* acetosyringone in the dark. Explants are then placed on selection medium [modified WPM + 1.0 mg/L thidiazuron (TDZ) + 0.5 mg/L α-naphthaleneacetic acid (NAA) + 10 mg/L kanamycin monosulfate (Km) + 250 mg/L cefotaxime] in the dark for 2 wk, followed by culture in the light at 30 $\mu E/m^2/s$ at 25°C. Proliferation of Km-resistant shoots is performed on WPM + 1.0 mg/L zeatin + 20 mg/L Km + 250 mg/L cefotaxime. The transformation protocol yields Km-resistant GUS- positive shoots that are also polymerase chain reaction (PCR) positive at frequencies, defined as the percentage of inoculated explants that produced GUS- and PCR-positive shoots, of 15.3% for Aurora, 5.0% for Bluecrop, 10.0% for Brigitta, and 5.6% for Legacy. Stable integration of *gusA* was confirmed by Southern hybridization *(11)*. Because all of the four cultivars tested can produce transgenic plants following the described protocol, it should enable future success on other blueberry genotypes and begin testing applicable transgenes.

2. Materials

2.1. Plant Material

1. Starting plant materials: One-yr-old softwood branches of highbush blueberry cvs. Aurora (Michigan State University cv.), Bluecrop, Brigitta, and Legacy.
2. Sterilizing solution: 30% Clorox® (v/v) + 0.02% (v/v) Tween 20.
3. Leaf explants are taken from in vitro cultured stock shoots.

2.2. Culture Medium and Stock Solutions

Media pH is adjusted with NaOH before adding the agar and autoclaving (*see* **Note 1**). Double distilled water (ddH_2O) is used unless otherwise mentioned. All media are autoclaved at 121°C for 20 min at 105 kPa and kept at room

temperature if they can be used within 1 wk; otherwise, they can be stored at 4°C up to 2 wk. Stock solutions of zeatin, Km, cefotaxime, timentin and acetosyringone are filter-sterilized through 0.22 μm Millipore filters (Millipore Corporation, Bedford, MA), and added to liquid medium with agar cooled to between 50 and 60°C or to liquid medium at room temperature after autoclaving.

1. Stock culture medium (WPM4Z) (*16*): McCown's woody plant basal salt mixture (WPM) (*15*) (Sigma, St. Louis, MO), Murashige and Skoog (MS) (*17*) vitamin solution (Sigma), 556 mg/L $Ca(NO_3)_2 \cdot 4H_2O$, 4 mg/L zeatin, 20 g/L sucrose, 6 g/L Bacto™ agar (Becton Dickinson and Company, Sparks, MD), pH 5.2. Pour in 25 × 150-mm glass culture tubes with caps (Sigma) (15 mL/tube) and/or 40 × 110 mm glass jars with caps (PhytoTechnology Lab., Shawnee Mission, KS) (30 mL/jar).
2. 100X stock solution of modified McCown's woody plant medium salts (MWPM): (A) 40 g NH_4NO_3, 68.4 g $Ca(NO_3)_2 \cdot 4H_2O$; (B) 17 g KH_2PO_4, 0.62 g H_3BO_3, 0.025 g $Na_2MoO_4 \cdot 2H_2O$; (C) 19 g KNO_3; (D) 37 g $MgSO_4 \cdot 7H_2O$, 2.23 g $MnSO_4 \cdot H_2O$, 0.86 g $ZnSO_4 \cdot 7H_2O$, 0.025 g $CuSO_4 \cdot 5H_2O$; (E) 7.34 g ethylenedinitrilotetraacetic acid (ferric sodium salt). Make up to 1 L with ddH_2O. Store at 4°C after autoclaving.
3. Regeneration medium (RM): MWPM salts, MS vitamins, 1 mg/L thidiazuron (TDZ), 0.5 mg/L α-naphthaleneacetic acid (NAA), 20 g/L sucrose, 6 g/L Bacto™ agar, pH 5.2. Pour 25 mL per dish (100 × 20 mm) (Corning Inc., Corning, NY) (*see* **Note 2**).
4. Co-cultivation medium (MWPM2S): MWPM salts, MS vitamins, 100 μM actosyringone, 20 g/L sucrose, 6 g/L Bacto™ agar (for solidified medium), pH 5.2. Pour agar medium into 100 × 20 mm dishes 25 mL/dish.
5. Selection medium: RM + 10 mg/L Km + 250 mg/L cefotaxime. Pour into 100 × 20 mm dishes 25 mL/dish.
6. Shoot proliferation medium for transformants: WPM4Z + 10 mg/L Km + 250 mg/L cefotaxime. Pour into 40 × 110 mm glass jars with caps (PhytoTechnology), 30 mL/jar.
7. *Agrobacterium* culture medium (YEB) (*18*): 1 g/L Bacto-yeast extract, 5 g/L beef extract, 5 g/L peptone, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 5 g/L sucrose, 15 g/L Bacto™ agar (for solidified medium), pH 7.0. After addition of 50 mg/L Km, pour the agar-containing medium into 100 × 15 mm Petri dishes (Falcon, Franklin Lakes, NJ), 25 mL/dish.
8. Stock solution of plant growth regulators: 1 mg/mL TDZ (PhytoTechnology) in dimethyl sulfoxide (DMSO); 1 mg/mL zeatin (PhytoTechnology) in solvent 1 *N* NaOH, dilute ddH_2O; 1 mg/mL NAA (Sigma). These stocks store at 4°C up to 6 mo.
9. Antibiotic stock solution: 50 mg/mL Km (PhytoTechnology); 250 mg/mL cefotaxime (PhytoTechnology); 250 mg/mL timentin (GlaxoSmithKline, Research Triangle Park, NC); the stocks are all dissolved in ddH_2O and stored in aliquots at –20°C.
10. 100 m*M* acetosyringone in DMSO; store in aliquots at 4°C.
11. Planting medium: Sphagnum peat moss (Fafard Peat Moss Co., Ltd., Inkerman, NB, Canada) already in plastic flats and soaked with tap water (12 paks × 6 cells/pak).
12. Nutrient solution for planting medium grown plants: 0.2 g/L fertilizer (nitrogen:phosphorus:potassium = 21:7:7) dissolved in tap water.

2.3. Bacterial Strains and Binary Vector

1. *A. tumefaciens* strain EHA105 containing the binary vector pBISN1. EHA105 culture is suspended in 20% sterile glycerol (v/v); store in aliquots at −80°C.
2. pBISN1 contains *npt*II conferring Km resistance and a plant intron interrupted GUS gene (*gusA*) driven by a chimeric promoter: $(Aocs)_3$AmasPmas *(13,14)*.

2.4. Histochemical GUS Assay and PCR

1. Stock solution of 5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylamine salt (X-Gluc) (PhytoTechnology): 20 mg/L in dimethyl formamide (DMF); store in the dark at −20°C.
2. Staining solution: 100 m*M* phosfate buffer, 100 μ*M* EDTA and 100 m*M* Triton X-100, 1 mg/L X-Gluc.
3. Dneasy Plant Mini or Maxi Kit (Qiagen Inc., Valencia, CA).
4. The primers corresponding to a 377-bp fragment of the coding region of *gus*A are 5′-GATCCTCGCATTACCCTTACGC-3′ and 5′-ATGAGCGTCGCAGAACATTAC-3′. 20 m*M* stock solution is stored at −20°C.
5. The primers for a 600-bp fragment of the *npt*II coding region *(19)* are 5′-GAGGC TATTCGGCTATGACTG-3′ and 5′-ATCGGGAGCGGCGATACCGTA-3′. 20 mM stock solution is stored at −20°C.
6. RED Taq™ ReadyMix™ PCR Reaction Mix with $MgCl_2$ (Sigma).

3. Methods

3.1. Establishment of Stock Cultures

1. Surface sterilize year-old softwood branches collected in June, trimmed to 10 to 15 cm in length, in 30% Clorox® + 0.02% (v/v) Tween-20 in 25 ×150 mm glass tubes for 20 min.
2. Rinse the branches three times (5 min/time) with sterile distilled water.
3. Cut the branches into 1 to 2 cm pieces each with a single bud.
4. Insert branch pieces individually into 25 × 150 mm glass tubes each containing 15 mL stock culture medium.
5. Incubate explants for 4 to 6 wk at 25°C, 30 $\mu E/m^2/s$ of 16-h d photoperiod.
6. Excise 1- to 5-cm long shoots, place 4 to 6 shoots horizontally on 30 mL stock culture medium in each 40 × 110 mm glass jar and incubate at 25°C, 30 $\mu E/m^2/s$ of 16-h d photoperiod from cool white fluorescent tubes.
7. Subculture the stock shoots at 6-wk intervals for 12 wk.

3.2. Preparation of Leaf Explants

1. Leaves from 4 to 8 cm long newly-formed in vitro shoots, excluding the 3 youngest leaves near the tip for each shoot, are the source of explants.
2. Leaf explants are excised from the distal $^2/_3$ of the blade using stainless steel, dissecting scissors (115 mm in length) (*see* **Note 3**).
3. The excised explants are placed on two sheets of MWPM2S-soaked sterile filter paper in a Petri dish (100 × 20 mm) to keep them moist.

3.3. Infection and Co-cultivation

1. Streak the *A. tumefaciens* strain EHA105: pBISN1 stock culture to a YEB plate using an inoculating loop (*see* **Note 4**).
2. Culture the plate for 3 d at 28°C.
3. Culture single colonies of the strain EHA105 in 10 mL of liquid YEB + 10 μL of 50 Km stock solution in a 50 mL Corning tube with constant shaking (300 rpm) in an incubator shaker at 28°C for 48 h. Do not over tighten the cap.
4. Measure the optical density (OD) of the bacterial culture at 600 nm.
5. Collect the bacterial cells by a 2 min centrifugation of 2 mL bacterial culture in a sterile 2 mL Eppendorf at 2500*g* at room temperature.
6. Discard the supernatant and suspend the pellet in liquid MWPM2S to an OD_{600} of 0.5 (*see* **Note 5**).
7. Incubate 20 mL suspension cells in a 50-mL Corning tube for 1 h at 28°C with constant shaking (300 rpm) in an incubator shaker.
8. Transfer the leaf explants to the 50-mL Corning tube containing 20 mL bacterial suspension cells.
9. Inoculate leaf explants in an incubator shaker at 28°C at 100 rpm for 1 h.
10. Pour off the bacterial suspension, transfer the explants onto two sheets of sterile Whatman filter paper in a 100 × 15 mm Petri dish. All bacterium contaminated waste should be autoclaved.
11. Blot dry and place the explants (80–100/dish) on solidified MWPM2S in a 100 × 15 mm dish. The dishes are sealed using truncated food wrap (2 cm in width) unless otherwise mentioned.
12. Co-cultivate leaf explants with EHA105 at 25°C for 6 d in the dark (*see* **Note 6**).

3.4. Selection and Regeneration

1. After co-cultivation, transfer the explants to a 50-mL Corning tube.
2. Wash the explants 2 times (5 min/time) in 50 mL liquid RM + 500 mg/L cefotaxime with constant shaking by hand (*see* **Note 7**).
3. Rinse 3 times (5 min/time) in 50 mL liquid RM without cefotaxime (*see* **Note 7**).
4. Blot dry the explants on sterile Whatman filter paper in a 100 × 15 mm petri dish.
5. Place the leaf explants (20/dish), abaxial side up, on selection medium (*see* **Note 8**). Ensure that explants are in a good contact with the medium.
6. Culture at 25 °C for 2 wk in the dark.
7. Transfer the plates to a 30 $\mu E/m^2/s$ of 16-h d photoperiod at 25°C.
8. Subculture the leaf explants on fresh selection medium at 3-wk intervals at 25°C, 30 $\mu E/m^2/s$ of 16-h d photoperiod; discard any dead explants during subcultures.

3.5. Regrowth

1. During subcultures, transfer selected Km-resistant shoot clusters individually onto 25 mL of WPM4Z + 250 mg/L cefotaxime + 10 mg/L Km in a 40 × 100 mm glass jar, culture at 25°C, 30 $\mu E/m^2/s$ of 16-h d photoperiod for 4 wk. Cefotaxime is used to control *Agrobacterium* growth, and Km at 10 mg/L is added to the medium as the selection agent.

2. Excise the elongated shoots, place an individual shoot horizontally on 30 mL WPM4Z + 250 mg/L cefotaxime + 10 mg/L Km in a 40 × 100 mm glass jar, label each jar accordingly, culture at 25°C, 30 μE/m^2/s of 16-h d photoperiod for 6 wk.
3. Subculture the shoots from each jar on WPM4Z + 250 mg/L cefotaxime + 10 mg/L Km following the method for stock culture at least twice.
4. During subculture, excise some leaves from Km-resistant shoots, culture the leaves on selection medium. Leaves from true transgenic shoots will regenerate new adventitious shoots (*see* **Note 9**).

3.6. Rooting

1. Autoclave sphagnum peat moss plus tap water at 121°C for 5 min at 105 kPa to soak the moss.
2. Fill cell trays made of 12 individual 6-paks with the soaked sphagnum moss, thoroughly water the sphagnum peat moss.
3. Excise the shoots (3–5 cm in length).
4. Insert individual shoots directly in sphagnum moss in 6-paks.
5. Cover the flats with transparent plastic covers, culture for 4 to 8 wk at 25°C and 30 μE/m^2/s of 16-h d photoperiod; water the plants at about 4-d intervals. At this stage plants are grown in a plant culture room.
6. Progressively open and remove the plastic covers in the first 7 d, water the plants at 2-d intervals, and apply nutrient solution three times per wk. The young plants continue growth for 3 to 4 wk after removing the cover.

3.7. Greenhouse Care

1. Fill 4-inch plastic pots with water soaked sphagnum peat moss (Fafard Peat Moss Co., Ltd.) and Suremix Perlite planting medium (Michigan Grower Products Inc., Galesburg, MI) (1:1 [v/v]).
2. Transplant the plants (10–15 cm in height) from the cell paks into 4-inch pots.
3. Transfer the plants to the greenhouse (21–32°C /10–21°C (d/night), 25 to 65 μE/m^2/s on a 10- to 14-h d photoperiod.
4. Water the plants as needed and fertilize weekly using a nutrient solution of 0.2 g/L 21:7:7 fertilizer.
5. Plants can be planted in the field in May in Michigan.

3.8. Identification of Transformed Shoots

Further screening and confirmation for transgenic shoots can be performed using histochemical GUS assay, PCR amplification, and Southern blot hybridization.

3.8.1. Histochemical GUS Assay

1. During subculture of Km-resistant shoots, excise part of the stem tissues from the base of the shoots, stain the tissues in GUS assay solution at 37°C overnight (*see* **Note 10**).

2. Plants or tissues of planting medium-grown transformants can also be stained in GUS assay solution at 37°C overnight (*see* **Note 11**).
3. After staining, the chlorophyll is removed with 70% (v/v) ethanol washes at 37°C.

3.8.2. PCR Amplification

1. Excised leaves from in vitro cultured plants or planting medium grown plants are used. Extract genomic DNA following the instruction of the DNeasy Plant Mini (for PCR) or Maxi (for Southern blot) Kit.
2. For each 20 μL PCR reaction, add between 150 and 200 ng of template DNA, 10 μL of RED Taq™ ReadyMix™ PCR Reaction Mix, 0.5 μL of forward primer, 0.5 μL of reverse primer, bring the volume to 20 μL using PCR grade water.
3. The reaction conditions for a 377-bp fragment containing the *gus*A coding region are 94°C for 2 min, 35 cycles of 94°C for 10 s, 55°C for 1.5 min and 72°C for 2 min, with a final 10 min extension at 72°C.
4. The reaction conditions for a 600-bp fragment of the *npt*II coding region include 94°C for 2 min, 35 cycles of 94°C for 30 s, 60°C for 1.5 min and 72°C for 3.5 min, with a final 10 min extension at 72°C ***(19)***.
5. The PCR reaction products are separated on 0.8% (w/v) agarose gel containing ethidium bromide and visualized under ultra-violet light.
6. Stable integration of the transgenes as well as the copy number of integration is confirmed using Southern blot hybridization (*see* **Note 12**).

4. Notes

1. The change of pH caused by the addition of Km, cefotaxime, and acetosyringone to media is usually not considered. However, addition of zeatin to the stock culture medium after autoclaving will increase the pH of the medium because of the solvent (1 *N* NaOH) in the zeatin stock solution. Thus, to get a final pH of 5.2 for stock culture medium, preliminary experiments should be performed to work out how much (x) the pH will increase after the addition of a certain amount of zeatin; then adjust the pH for stock culture medium to $5.2 - x$ prior to autoclaving. x is variable to different stock solutions of zeatin.
2. RM has led to the highest number of regenerated shoots per explant for all six highbush blueberry cultivars tested although the shoot regeneration efficiencies and patterns vary among the cultivars ***(11)***. There are two regeneration patterns among the cultivars tested. Shoot regeneration of cvs. Aurora and Brigitta occurs predominantly on the whole surface of the leaf explants (*see* **Fig. 1A,B**). For cvs. Bluecrop and Legacy, most shoots are usually formed from the cut edges of the leaf explants (*see* **Fig. 1C,D**).
3. Using small dissecting scissors was found to be very effective for excising leaf explants; a quick cutting, thus, can shorten the exposure of the leaf explants in the airflow of the sterile cabinet.
4. Despite successful transformations using agropine strain EHA105, leaf explants of blueberry cultivars were also found susceptible to octopine strain LBA4404 ***(20)*** and nopaline strain GV3101 ***(21)***, and both *A. tumefaciens* strains may be used for stable transformation.

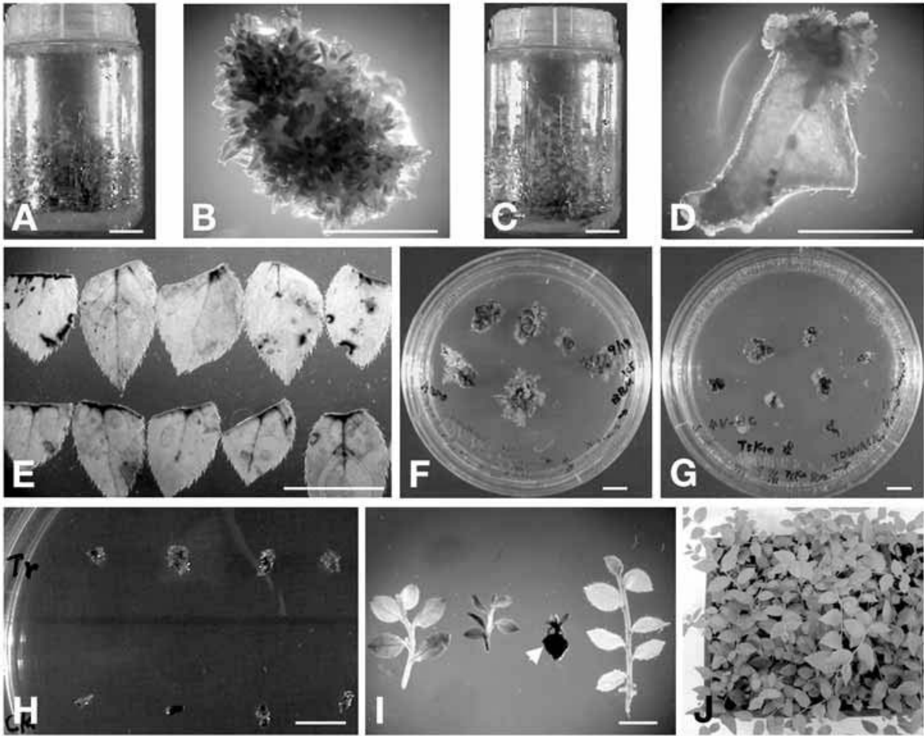

Fig. 1. Transformation of blueberry cultivars Aurora and Bluecrop. **(A)** Stock cultures of cv. Aurora. **(B)** Shoot regeneration for cv. Aurora on RM. **(C)** Stock culture of cv. Bluecrop. **(D)** Shoot regeneration for cv. Bluecrop on RM. **(E)** Transient GUS expression in leaf disks of cv. Aurora (*top*) and cv. Bluecrop (*bottom*) after 6-d co-cultivation. **(F)** Production of kanamycin-resistant shoots from inoculated leaf disks of cv. Aurora after 10-wk selection. **(G)** Production of kanamycin-resistant shoots from inoculated leaf disks of cv. Bluecrop after 10-wk selection. **(H)** Regrowth of shoots from transgenic Aurora leaves (*top*) and nontransformed control (*bottom*) after 10 wk on RM + 10 mg/L Km. **(I)** GUS staining in transgenic tissues of cv. Aurora; *arrow* indicates strong blue staining at the base of a shoot cluster. **(J)** Transgenic plants growing in planting medium; bars = 1 cm.

5. This step is used to remove the liquid YEB. The Km 50 mg/L in YEB is toxic to leaf explants.
6. Blueberry leaf explants are not hypersensitive to *A. tumefaciens*. After 6-d co-cultivation, there is little tissue necrosis, and transient GUS expression is detectable by staining (*see* **Fig. 1E**). Proper co-cultivation medium and 100 µ*M* acetosyringone help promote gene transfer.
7. After washes, most of the agrobacterial cells on the explant surfaces are removed; thus, these washes make it possible to use a lower concentration of cefotaxime

(250 mg/L) for successful inhibition of bacterial growth in the following selection steps.

8. Leaf explants of blueberry cultivars are very sensitive not only to Km ***(8,11)*** but also to glufosinate ammonium. In our recent transformation studies using the chimeric bialaphos resistance (*bar*) gene as selectable marker, 0.1 mg/L glufosinate ammonium in RM was found to inhibit shoot regeneration from nontransformed leaf explants, and transgenic plants have been obtained after selection with 0.1 mg/L glufosinate ammonium plus 250 mg/L timentin. The high level of sensitivity of leaf explants of blueberry indicates that dose experiments should be performed when other selectable markers are proposed. Compared with cefotaxime, timentin is less expensive. When timentin is used for stable transformation in our recent researches, transgenic plants can also be regenerated.
9. When selection is done at 10 mg/L, Km resistant shoot clusters can be obtained from all 4 cvs. (*see* **Fig. 1F,G**). Moreover, leaves from transgenic plants can also regenerate into shoots on RM containing Km 10 mg/L, whereas leaves from nontransformed shoots die in 8 wk (*see* **Fig. 1H**).
10. At this stage, tissues from the base of Km-resistant shoot clusters are more suitable for GUS staining. In the top 25% leaves of a 4 to 5 cm long shoot, blue staining is usually weak although the leaves near the base show strong blue staining (*see* **Fig. 1I**). No blue GUS staining will be observed in agrobacterial cell of EHA105: pBISN1 due to the plant intron in the *gus*A.
11. When soil-grown transgenic plants with 10 to 15 leaves (*see* **Fig. 1J**) are stained in GUS assay solution, blue staining can be observed in roots and stems, but not in the leaves. In contrast, nontransgenic plants do not show blue staining.
12. Low-copy-number (1–4) transgenic blueberry plants can be obtained using *A. tumefaciens*-mediated transformation.

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Grapevine (*Vitis vinifera* L.)

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Summary

Grapevine (*Vitis*) is considered to be one of the major fruit crops in the world based on hectares cultivated and economic value. Grapes are used not only for wine but also for fresh fruit, dried fruit, and juice production. Wine is by far the major product of grapes and the focus of this chapter is on wine grape cultivars. Grapevine cultivars of *Vitis vinifera* L. have a reputation for producing premium quality wines. These premium quality wines are produced from a small number of cultivars that enjoy a high level of consumer acceptance and are firmly entrenched in the market place because of varietal name branding and the association of certain wine styles and regions with specific cultivars. In light of this situation, grapevine improvement by a transgenic approach is attractive when compared to a classical breeding approach. The transfer of individual traits as single genes with a minimum disruption to the original genome would leave the traditional characteristics of the cultivar intact. However, a reliable transformation system is required for a successful transgenic approach to grapevine improvement. There are three criteria for achieving an efficient *Agrobacterium*-mediated transformation system: (1) the production of highly regenerative transformable tissue, (2) optimal co-cultivation conditions for both grapevine tissue and *Agrobacterium*, and (3) an efficient selection regime for transgenic plant regeneration. In this chapter, we describe a grapevine transformation system which meets the above mentioned criteria.

Key Words: Grapevine; *Vitis*; embryogenic callus; *Agrobacterium*; selectable markers; transgenic; antibiotic sensitivity; reporter genes; plant regeneration; transformation efficiency.

1. Introduction

Most of the known grapevine wine varieties have been vegetatively propagated for several centuries. The reasons for the persistence of traditional European grapevine (*Vitis vinifera* L.) cultivars for wine production are many with both plant and human factors involved ***(1)***. All *V. vinifera* cultivars are

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highly heterozygous and do not breed true from seed. The combination of genes in a heterozygous genome responsible for wine quality is conserved by vegetative propagation. Thus, classical breeding programs, particularly those that have attempted to improve disease resistance and maintain wine quality have had limited success in the past. Consumer pressure for the same cultivars and the specie's lack of amenability to classical breeding has focused research attention on producing improved transgenic plants of established cultivars as the approach causes minimum disturbance to the original heterozygous genome (*2*). Moreover, the ability to produce transgenic plants is also an invaluable tool for understanding gene function and biological processes in grapevine (*3*).

A reliable transformation system is required for a successful transgenic approach to grapevine improvement especially for disease and stress resistance or tolerance (*4*). There are several prerequisites for achieving an efficient *Agrobacterium*-mediated transformation system.

First, the production of highly regenerative transformable tissue is critical. Since the first report of successful grapevine transformation (*5*), the methods for the production of transgenic grape plants have been based on the use of embryogenic cultures (*6*). Previously, the lack of success in combining *Agrobacterium*-mediated transformation with direct shoot organogenesis from leaf explants was explained by the fact that the cells competent for regeneration were not competent for transformation (*7*). However, recent work indicates that techniques based on regeneration by organogenesis can be efficient (*8*). The explant source, type and quality of embryogenic cultures are a key factor in successful transformation and improved conditions for initiation and maintenance of cultures suitable for genetic transformation have been defined (*9*).

Second, it is important to optimize co-cultivation conditions for both *Agrobacterium* strains and grapevine tissue. The infection of cells with any given *Agrobacterium* strain and successful T-DNA integration is affected by several factors, such as strain, bacterial culture conditions, bacterial density, co-cultivation time, and media used.

Finally, one needs an efficient selection regime for transgenic plant regeneration. The use of the selectable marker gene *npt*II that induces resistance to kanamycin has been widely reported in transformation experiments in *Vitis*. As an alternative to the *npt*II gene, the *hpt* gene has also been used efficiently with hygromycin as the selective agent (*10–12*). The *bar* (*pat*) gene encoding phosphinothricin acetyl transferase (PAT) has also been used with the herbicide Basta® as the selective agent (*11*) but its efficiency is debated (*13,14*). In addition initial attempts to use the phosphomannose-isomerase (*pmi*) gene as an alternate selectable marker have been reported as disappointing (*13,15*).

The transformation procedure described below uses embryogenic cultures obtained from immature anthers. This tissue type is widely used to induce

somatic embryogenesis in grapevine ***(11,24,26)***. Transformation can also be used with embryogenic cultures obtained from other tissues such as ovaries, nucellus, embryos, hypocotyls, or young leaves from in vitro plantlets. Maintenance of embryogenic cultures in a state suitable for transformation appears dependent on the interaction of genotype and culture medium, and in many cases represents a greater challenge than the initiation step ***(2)***. Most importantly, two distinct types of embryogenic cultures are usually obtained on semi-solid media: a culture designated Type I which consists mostly of small globular embryos and undifferentiated calli and a Type II culture which often develops from Type I calli and consists entirely of somatic embryos at various stages of development from heart shape to torpedo stage, which proliferate by secondary embryogenesis ***(12)***.

When the response of different culture types to *Agrobacterium tumefaciens* transformation was examined, it was found that culture type did not have a major effect on initial rates of transformation. This can be determined by the level of green fluorescent protein (GFP) in cell clusters after co-cultivation (*see* **Fig. 1C**) and measured by the number of positive units recorded/plate ***(17)***. However, different culture types have a significant effect on the recovery of transgenic plants, with more plants recovered from Type I cultures ***(12)***. The reasons for the difference in recovery rate are complex. In *V. vinifera*, the data for optimal transformation and selection conditions are conflicting as they may not only result from differences in genotypes and selection strategies, but also from the embryogenic state of the cultures at the time of transformation. The number of transgenic plants which can be recovered from an experiment is important when evaluating transgene expression. Aberrant expression patterns of a transgene under the control of a constitutive promoter such as CAMV35S can reach a frequency of 35% in transgenic plants evaluated ***(26)*** and need to be taken into account when seeking to introduce a trait which has commercial potential.

With the procedure described below, the number of embryos growing and rooting on germination medium under kanamycin selection, and showing *uidA* or *gfp* expression is variable, depending on the cultivar and the *A. tumefaciens* strain. From 1 g of co-cultivated embryogenic calli, it may range from 10 to 100 or more embryos. A bottleneck in the transformation procedure still remains at the stage of shoot and plantlet development. The percentage of transgenic plantlets regenerated from transgenic embryos using this procedure ranges from 10 to 33%, depending on the cultivar. Among these transgenic plants, those regenerated from independent transformation events, as determined by Southern Blot analysis range from 77 to 100%. The final transformation efficiency obtained with the procedure described below can range from 1 to 33 or more independent transgenic lines obtained from 1 g of embryogenic calli. For

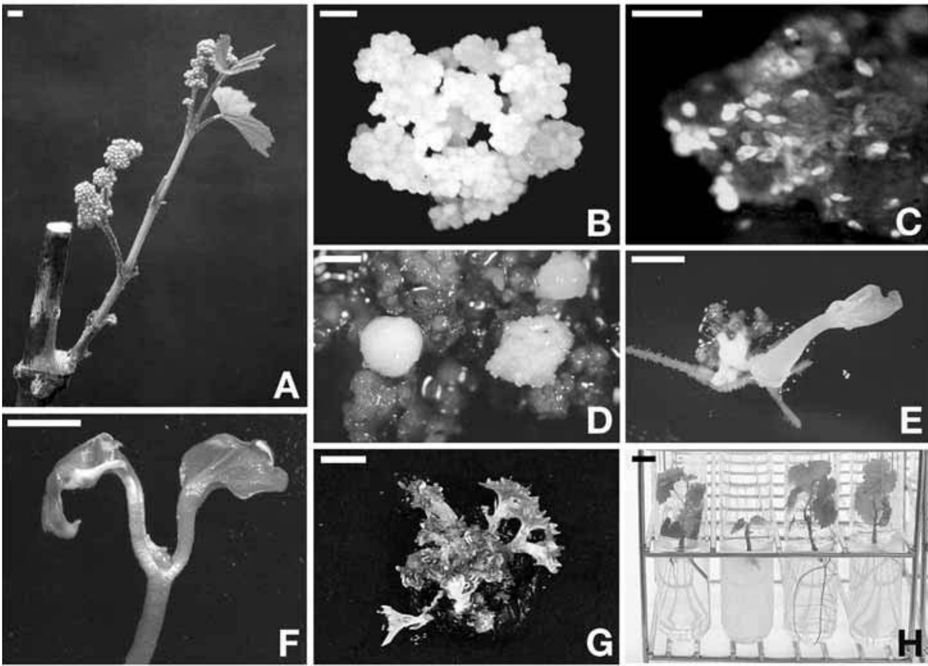

Fig. 1. Successive stages of the transformation procedure in grapevine. **(A)** Inflorescence with immature flower buds produced on cutting and ready for anther culture; bar = 5 mm). (**B**) Embryogenic calli grown on GS1CA medium prior to co-cultivation; bar = 1 mm. (**C**) Bright clusters of *gfp*-expressing cells observed 5 wk after co-cultivation; bar = 50 µm. (**D**) Clusters of kanamycin resistant embryogenic cells visually selected among dead tissue two months after co-cultivation; bar = 1 mm. (**E**) Germination of a kanamycin resistant embryo on MG1 medium; bar = 5 mm. (**F**) Well-developed embryo before trimming of cotyledons and roots; bar = 5 mm. (**G**) Growth and axillary branching of the embryo apical meristem on BFe2 medium with 50 µg/mL kanamycin; bar = 5 mm. (**H**) Rooting of transformed plantlets on GNBC medium with 50 µg/mL kanamycin. *Note*: Second plant from the left is an untransformed plantlet.

wine grape cultivars, some of them—such as Chardonnay—are easy to transform *(26)*. Others—such as Pinot Noir—are more recalcitrant despite it being a parent of Chardonnay *(27)*.

2. Materials

2.1. Plant Material

Somatic embryogenic cultures are initiated from immature anthers from many grapevine cultivars including Sultana, Portan, Shiraz, Chardonnay, Cabernet Sauvignon, Riesling, and Sauvignon Blanc.

2.2. *Agrobacterium Strains*

The *A. tumefaciens* strain EHA101 (*16*) or its derivative EHA105 that contain a binary plasmid are used for transformation (*see* **Note 1**). They are more efficient for grapevine transformation than the widely used strain LBA4404 and other strains (*17*).

2.3. *Culture Media for* A. tumefaciens

Media are sterilized by autoclaving for 30 min at 110°C.

1. Modified MG/L medium (*18*): 5 g/L of mannitol, 1 g/L of L-glutamate, 5 g/L of tryptone, 2.5 mL/L of Fe-ethylenediamine tetracetic acid (EDTA) stock solution, 5 g/L of NaCl, 150 mg/L of KH_2PO_4, 100 mg/L of $MgSO_4 \cdot 7H_2O$, 2.5 g/L yeast extract, 20 µg/L of biotin, and antibiotics depending on the bacterial strain and binary vector (100 µg/mL kanamycin and 25 µg/mL rifampicin for EHA105/pBINm-gfp5-ER). Adjust to pH 7.0 with NaOH.
2. Induction medium: ABB salts (*19*) (20 g/L of NH_4Cl, 12.3 g/L of $MgSO_4 \cdot 7H_2O$, 3.0 g/L of KCl, 0.265 g/L of $CaCl_2 \cdot 2H_2O$, 0.5 g/L of $FeSO_4 \cdot 7H_2O$, 5 µg/L of biotin), 2 m*M* NaH_2PO_4 at pH 5.6, 40 m*M* 2-(*N*-morpholino) ethanesulfonic acid (MES), 0.5% glucose and 100 µ*M* acetosyringone.

2.4. *Stock Solutions and Other Supplies*

All stock solutions were sterilized through a 0.2 µm filter and stored at 4°C unless otherwise stated.

1. 10X Half strength Murashige and Skoog (MS) macroelements (*22*): 16.5 g/L of NH_4NO_3, 4.4 g/L of $CaCl_2 \cdot 2H_2O$, 3.7 g/L of $MgSO_4 \cdot 7H_2O$, 19.7 g/L of KNO_3, and 1.7 g/L of KH_2PO_4.
2. 10X NN macroelements (*20*): 7.2 g/L of NH_4NO_3, 9.5 g/L of KNO_3, 4.4 g/L of $CaCl_2 \cdot 2H_2O$, 3.7 g/ L of $MgSO_4 \cdot 7H_2O$, and 1.7 g/L of KH_2PO_4.
3. 10X GNBC macroelements (*25*): 10 g/L of KNO_3, 5 g/L of $Ca(NO_3)_2 \cdot 4H_2O$, 1.6 g/L of NH_4NO_3, 1.25 g/L of $MgSO_4 \cdot 7H_2O$, and 1.25 g/L of KH_2PO_4.
4. 1000X MS microelements (*22*): 6.2 g/L of H_3BO_3, 22.3 g/L of $MnSO_4 \cdot 4H_2O$, 8.6 g/L of $ZnSO_4 \cdot 7H_2O$, 0.83 g/L of KI, 0.25 g/L of $Na_2MoO_4 \cdot 2H_2O$, 25 mg/L of $CuSO_4 \cdot 5H_2O$ and 25 mg/L of $CoCl_2 \cdot 6H_2O$.
5. 1000X GNBC microelements (*25*): 460 mg/L of $MnSO_4 \cdot 4H_2O$, 250 mg/L of KI, 58 mg/L of $ZnSO_4 \cdot 7H_2O$, 25 mg/L of H_3BO_3, 25 mg/L of $CuSO_4 \cdot 5H_2O$, 25 mg/L of $NiCl_2 \cdot 6H_2O$, 25 mg/L of $CoCl_2 \cdot 6H_2O$, and 25 mg/L of $Na_2MoO_4 \cdot 2H_2O$.
6. 200X Fe-EDTA (*22*): Dissolve 7.44 g of $Na_2EDTA \cdot 2H_2O$ in 900 mL of nanopure water. Heat the solution to almost boiling point and gradually add 1.86 g of $FeSO_4 \cdot 7H_2O$. Make up to 1 L with nanopure water.
7. 200X citrate ferric: Dissolve 4 g ammonium ferric citrate in 1000 mL nanopure water.
8. 1000X vitamins B5 (*21*): 100 g/L of mesoinositol, 10 g/L of thiamine HCl, 10 g/L of nicotinic acid, 1 g/L of pyridoxine HCl.
9. 1000X vitamins T (*23*): 50 g/L of mesoinositol, 1 g/L of nicotinic acid, 1 g/L of thiamine HCl, 1 g/L of pyridoxine HCl, 1 g/L of calcium pantothenate, and 0.01 g/L of biotin.

10. 1000X amino acid mix *(24)*: 100 g/L of glutamine, 10 g/L of phenylalanine, and 2 g/L of glycine.
11. 1 m*M* 2,4-dichlorophenoxyacetic acid (2,4-D): Dissolve 44.2 mg in 1 mL 10 *N* NaOH. Add nanopure water and heat until completely dissolved. Make up the volume to 200 mL in nanopure water.
12. 1 m*M* 6-benzylaminopurine (BAP): Dissolve 45.04 mg in 1 mL of 10 *N* NaOH. Add nanopure water to make a volume of 200 mL.
13. 1 m*M* β-naphthoxyacetic acid (NOA): Dissolve 40.44 mg in 1 mL 10 *N* NaOH. Add nanopure water to make a volume of 200 mL.
14. 1 m*M* indole-3-acetic acid (IAA): Dissolve 35.0 mg in 1 mL 10 *N* NaOH. Add nanopure water to make a volume of 200 ml.
15. Timentin® (Smith-Kline Beecham, Boronia, Australia): Dissolve in nanopure water at a concentration of 250 mg/mL. Store as aliquots at –20°C.
16. Kanamycin monosulfate: Dissolve in nanopure water at a concentration of 100 mg /mL. Store as aliquots at –20°C.
17. Rifampicin: Dissolve in dimethyl sulfoxide (DMSO) at a concentration of 25 mg /mL. Store as aliquots at –20°C.
18. Acetosyringone: Dissolve in absolute ethanol at a concentration of 100 m*M*. Store as aliquots at –20°C.

2.5. Plant Material and Tissue Culture

All the media are sterilized by autoclaving for 30 min at 110°C.

1. Culture medium for initiation of embryogenic calli from anthers (PIV): NN macroelements *(20)*, MS microelements, Fe-EDTA, vitamins B5, 4.5 µ*M* 2,4-dichlorophenoxyacetic acid (2,4-D), 8.9 *M* BAP, 60 g/L sucrose, and 3 g/L Phytagel® (Sigma) as the gelling agent; adjust pH to 5.7 with 1 *M* KOH.
2. Culture medium for maintenance of embryogenic calli from anthers (C_1P): Half strength MS macroelements, MS microelements, Fe-EDTA, vitamins T, amino acid mix with 1 g/L casein hydrolysate, 5 µ*M* 2,4-D, 1 µ*M* BAP; 30 g/L sucrose; and 5 g/L Phytagel® (Sigma); adjust pH to 6.0 with 1 *M* KOH. (*see* **Note 2**).
3. Culture medium to stimulate the formation of somatic embryos (GS1CA): NN macroelements, MS microelements, Fe-EDTA, vitamins B5, 10 µ*M* NOA, 20 µ*M* IAA, 1 µ*M* BAP, 60 g/L sucrose, 2.5 g/L activated charcoal, and 10 g/L bacto-agar; adjust pH to 5.7 with 1 *M* KOH.
4. Liquid co-cultivation medium (LCM): GS1CA medium without growth hormones, bacto-agar, and activated charcoal with 100 µ*M* acetosyringone.
5. Co-cultivation medium (CM): GS1CA medium added with 100 µ*M* acetosyringone.
6. Culture medium for the germination of embryos (MG_1): Half strength MS macroelements, MS microelements, Fe-EDTA, vitamins B5, 30 g/L sucrose, and 7 g/L bacto-agar.
7. Culture medium to stimulate the further development and greening of embryos (MG_2): MG_1 with 2 µ*M* BAP.
8. Medium to stimulate the axillary branching from caulinar meristems of germinating embryos (BFe2): Half-strength MS macroelements, MS microelements,

Fe-EDTA increased twofold, vitamins T, 4.4 μ*M* BAP, 20 g/L sucrose, and 7 g/L agar; adjust pH to 6.0 with 1 *M* KOH.

9. Medium to induce and stimulate the rooting of shoots (RIM): Half-strength MS macroelements, MS microelements, Fe-EDTA, vitamins T, 5 μ*M* IAA, 30 g/L sucrose, and 7 g/L bacto-agar; adjust pH to 6.0 with 1 *M* KOH.
10. Medium to micropropagate grapevines by nodal bud culture (GNBC): Macroelements and microelements ***(25)***, ferric citrate, vitamins T, 15 g/L sucrose; and 7 g/L bacto-agar; adjust to pH 6.5.

2.6. Other Reagents, Solutions, and Supplies

1. Flower surface disinfectant: 3.3% (w/v) calcium hypochlorite solution, corresponding to 1.2% active chlorine, and containing 0.1% (v/v) Tween-20 as wetting agent (*see* **Note 3**).
2. Soil mixture for the growth of transgenic plants: peat moss, compost and sand with a ratio of 1:1:1 (v/v/v).
3. Millipore® 100-μm nylon net filter (St. Quentin-Yveline, France).
4. Corning® 50 mL centrifuge tube (Corning Incorporated, NY).
5. Whatman No. 1 filter paper (Whatman, Springfield Milland, UK).
6. Magenta™ GA7-3 vessels (Life Technologies).

3. Methods

3.1. Initiation and Maintenance of Embryogenic Cultures

1. Hardwood cuttings from certified virus-free mother vines are stored at 4°C until required. Plant cuttings are placed in pots containing perlite in a controlled environment chamber (25°C, 16 h photoperiod with 50 μE/m^2/s) or greenhouse.
2. Immediately after bud burst, gently remove the basal leaves of the primary shoots with small forceps to promote the retention and growth of the inflorescences ***(28)***. Collect these inflorescences when they bear small (2–3 mm) unpollinated flower buds (*see* **Fig. 1A** and **Note 4**).
3. Store the inflorescences in Petri dishes with moist cotton wool for 3 d at 4°C in the dark (*see* **Note 5**).
4. Immerse flower buds for 30 s in 70% ethanol and further for 10 min in surface disinfectant. Rinse three times with sterile distilled water.
5. Dissect the flower buds under a stereo microscope in a laminar flow hood. Separate the calyptra from the peduncle with sharp glass rods or forceps and needles. Gently remove the anthers with their filaments (*see* **Note 6**).
6. Place about 30 anthers (6 flower buds) in 55 mm Petri dish containing PIV medium, taking care to put the anther with the filament in contact with the medium. Incubate at 28°C in the dark for 4 wk.
7. Transfer yellow-white emerging calli to fresh C_1P medium. Subculture every 4 wk selecting visually for friable, off-white calli containing proembryogenic clusters.
8. One month before the transformation experiment, transfer the proembryogenic calli to GS1CA medium (*see* **Fig. 1B**).

3.2. *Agrobacterium Culture Preparation*

1. Incubate a single colony of *Agrobacterium* containing a binary vector with a plant selectable marker gene for kanamycin resistance overnight at 28°C with shaking in 50 mL of modified MG/L medium with rifampicine 25 µg/mL and kanamycin 100 µg/mL, or other appropriate antibiotics according the plasmid properties.
2. Centrifuge the culture at 2600*g* for 5 min, resuspend the pellet in 100 mL induction medium and incubate for a further 2 h, with shaking at 100 rpm.
3. Centrifuge the culture as above and re-suspend the pellet in LCM medium and adjust the concentration of the bacterial suspension to an OD_{550} of 0.4 (approx 10^6 cfu/mL).

3.3. *Co-Cultivation of Embryogenic Callus With Agrobacterium*

1. Add 20 mL of the bacterial suspension to each gram of embryogenic calli in a 50 mL Corning centrifuge tube and then shake vigorously for 1 to 2 s.
2. After a 10 min incubation at 25°C, separate the calli with gentle shaking from the liquid phase with a 100 µm 3M nylon net filter.
3. Briefly blot on sterile Whatman filter paper and transfer onto a 90 mm Petri dish containing CM medium (*see* **Note 7**). Seal the Petri dishes with Parafilm® to prevent the culture from drying out and incubate in the dark at 22°C for 2 d.
4. After co-cultivation, wash the embryogenic calli in a 50 mL Corning tube with 20 mL LCM medium plus 1000 µg/mL Timentin®.
5. Retrieve the calli with a 100 µm nylon net and blot briefly on filter paper. Gently fragment the calli with forceps and distribute evenly onto 55 mm Petri dishes containing GS1CA medium with 1000 µg/mL Timentin. Incubate cultures in the dark at 28°C for 3 wk (*see* **Note 8**).

3.4. *Selection of Transgenic Embryos and Regeneration of Transgenic Plants*

1. Transfer calli onto 55 mm Petri dishes containing GS1CA medium with 1000 µg/mL timentin and 100 µg/mL kanamycin (*see* **Note 9**).
2. Subculture the calli onto fresh selective medium every 4 wk and gradually increase the kanamycin concentration to 150 µg/mL (*see* **Note 10**). Take care to remove dead tissue from the live clusters of kanamycin resistant embryogenic cells whilst subculturing (*see* **Fig. 1D**).
3. After two or three subcultures on selection medium, spread the calli thinly onto 90 mm plates containing MG_1 medium plus 150 µg/mL kanamycin and 1000 µg/mL timentin. Incubate plates at 28°C in the dark.
4. Transfer torpedo stage and further developed embryos (*see* **Fig. 1E**), to MG_2 medium and incubate under light (approx 60 µE/m^2/s) for 7 to 10 d at 25°C, until they turn green. Transfer the green embryos to MG_1 medium and incubate under lower light (approx 45 µE/m^2/s) for 2 wk.
5. Cut the root and trim cotyledons from the apical zone of the hypocotyls of well developed embryos. Place this trimmed embryo onto BFe2 medium plus 50 µg/mL kanamycin to stimulate growth of the shoot from the shoot meristem. Incubate at 25°C under attenuated light (approx 15 µE/m^2/s) (*see* **Fig. 1F**).

6. Subculture emerging shoots two or three times on the same medium and the same conditions to encourage axillary branching of the caulinar meristem (*see* **Fig. 1G**).
7. Regenerate whole plants by transferring shoots onto root induction medium (RIM) in Magenta™ GA7-3 vessels.
8. Select against chimeric plantlets by transferring nodal bud microcuttings of putative transformants onto GNBC medium with 50 µg/mL kanamycin (*see* **Fig. 1H** and **Note 11**). Generally, two subcultures are enough to discard any chimeric plantlets.
9. Test by polymerase chain reaction (PCR) well rooted kanamycin resistant plantlets for the presence of the gene of interest.
10. Subculture transgenic plantlets onto GNBC medium without kanamycin for in vitro conservation (5 plants/line) and acclimatization.

3.5. Transplanting and Greenhouse Care

1. Transfer the resulting healthy well-rooted plantlets into 10-cm diameter pots containing the soil mixture. Cover the pots with plastic bags and maintain under in vitro culture conditions for 3 wk.
2. Alternatively, transfer young plantlets with two or three leaves and one or two rootlets directly from agar medium to peat Jiffy pots 90 × 50 mm containing Perlite irrigated with liquid GNBC medium.
3. Place Jiffy pots in mini-glasshouses on a polystyrene base (45 pots/mini-glasshouse) and incubate at high humidity under in vitro culture conditions for 2 to 3 wk.
4. Transfer the mini-glasshouses to the greenhouse and progressively open to acclimatize the plants to low-hygrometric conditions. Transfer each Jiffy pot to a 10 cm diameter pot containing the soil mixture for further plant growth (*see* **Note 12**). Commercial nutrient solution (NPK + Fe + microelements) is brought to the pots during irrigation. Special care must be taken against development of Botrytis and other molds. The average rate for successful transplanting and acclimatization depends on the variety but is generally higher than 90%.
5. When the transgenic plants are successfully transplanted and well-growing in the greenhouse, confirm the integration of the gene of interest in the plant genome by Southern blot and analyze its expression of the gene by Northern blot, Western blot, or any other assays.

4. Notes

1. The T-DNA of the binary plasmid contains the gene of interest and a selectable marker such as *npt*II or *hpt*. The reporter gene *gfp* is currently used to check the effect of different factors such as cultivar, medium, strain, state of the calli, and conditions of co-culture on transformation efficiency. The effects these factors have on transformation efficiency are assessed by recording the amount of *gfp* expression in cells. The data collected correspond generally to the number of GFP positive units (individual cells or multi-cellular aggregates exhibiting GFP) recorded per plate 3 and 5 wk after co-cultivation.

2. The medium used to induce and maintain embryogenic calli cultures initiated from anthers can be successfully applied to induce and maintain embryogenic calli from leaf explants (*23*).
3. Sodium hypochlorite solution with the same percentage of active chlorine can be used in place of calcium hypochlorite. Solutions can be stored overnight but for a maximum of 2 d.
4. This technique can be applied throughout the year with greenhouse material. Alternatively, inflorescences can be collected in the vineyard at about 12 to 14 d before anthesis.
5. Chilling the inflorescences was found to improve the formation of embryogenic calli but the efficiency depends greatly on the genotype of the cultivar.
6. Embryogenic calli develop from anther filament tissue that is attached to the anther and will also develop from the filament scar on the anther if the filament is removed and this region of the anther is placed in contact with the medium. The developmental stage of the anther has a major effect on embryogenic response. Generally, the higher level of somatic embryogenesis occurs when the anther is at the tetrad or the uninucleate pollen stage, which corresponds to an opalescent color of the anther. Anthers that are translucent or have become yellow should not be used as they are less likely to produce embryogenic calli.
7. It can be useful to put a disk of Whatman filter paper on CM medium during co-cultivation to avoid over-growth of the bacteria, particularly with the super virulent EHA105 strain.
8. The use of an appropriate antibiotic, which can kill or suppress the growth of *Agrobacterium* is very important. Other antibiotics, such as cefotaxime at concentrations varying from 200 to 500 µg/mL (*8,10,29*), or carbenicillin at 500 µg/mL (*13,30,31*), can be used to kill the bacteria after co-cultivation. The optimum dose not only varies with the bacterial strain, but also with the cultivar and should be such that it inhibits *Agrobacterium* growth but allows for the normal regeneration of putative transgenic tissue.
9. Paromomycin sulfate (Sigma) can also be used as a selective agent with the *npt*II gene, at an initial concentration of 5 µg/mL, after co-cultivation and then increased gradually to 30 µg/mL (*11,32*). If the *hpt* gene is used as the selectable marker then hygromycin B (Sigma) can be used after co-cultivation at a concentration of 16 µg/mL (*10*) or 25 µg/mL (*12*), or with an initial concentration of 15 µg/mL increasing gradually up to 25 µg/mL (*11*).
10. Frequent sub-culturing to selective medium is an important step to maintain an optimum concentration of fresh antibiotic and the removal of compounds excreted by the dead cells.
11. This step represents an effective screen for identifying transgenic plantlets prior to potting out. An MS based medium or any other medium without growth hormones, is suitable for the micropropagation of grapevine by nodal bud culture. The GNBC medium is currently used in France to maintain in vitro cultures of several hundreds of cultivars, species, and hybrids in a germplasm repository (*33*). This demonstrates its versatility across a large range of grapevine genotypes.

12. A limited amount of information is available on the biological behavior of grapevine plantlets that are extremely sensitive to environmental stress during acclimatization ***(34)***. Although most grapevine laboratories have a simple, rapid and inexpensive acclimatization method no common protocol has been adopted.

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26

Strawberry (*Fragaria* × *ananassa*)

Bruno Mezzetti and Elisa Costantini

Summary

Genetic transformation in strawberry (*Fragaria* spp.) can be achieved by using the *Agrobacterium*-mediated procedure on leaves from in vitro proliferated shoots. Regardless of the sufficient regeneration levels achieved from leaf explants of some commercial strawberry genotypes, the regeneration of transformed strawberry plants remains difficult and seems to be strongly genotype dependent. In fact, the main factors that play an important role in the success of strawberry genetic transformation are the availability of both an efficient regeneration protocol and an appropriate selection procedure of the putative transgenic shoots.

The strawberry genetic transformation protocol herein described relates to two genotypes resulting from our experience with the highest regeneration and transformation efficiency. The study includes an octoploid *Fragaria* × *ananassa* cultivar (Sveva) and a diploid *F. vesca* cultivar (Alpina W.O.). All the different steps related to the leaf tissue *Agrobacterium* infection, co-culture, and selection of regenerating adventitious shoots, as well as the following identification of selected lines able to proliferate and root on the selective agent (kanamycin), will be described.

Key Words: Strawberry; leaf tissue regeneration; *Agrobacterium* infection; kanamycin selection.

1. Introduction

The *Agrobacterium tumefaciens*–mediated transformation represents one of the most common techniques of recombinant DNA, and it is largely employed to obtain genetically modified plants. Efficient transformation and regeneration methods are a priority for successful application of recombinant DNA technology to vegetative propagated plants such as strawberry.

To date, the most resourceful plant differentiation process for recombinant DNA technology in strawberry remains adventitious shoot organogenesis directly from somatic tissue or a previous callus formation. However, detailed

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developmental and physiological characterization of the whole sequence of the organogenic processes in strawberry somatic tissues is still mainly lacking (*1*). The genotype and type of explants represent important factors affecting the regeneration process and, consequently, the genetic transformation efficiency. For several *Fragaria* × *ananassa* genotypes, efficient regeneration protocols have been identified by using different types of somatic tissues (*2–6*), although leaf tissue has been the most studied in several regeneration experiments (*3,7,8*) and in most cases it has displayed the highest efficiencies for high regeneration frequencies (*6*). Leaf explants have also been particularly useful for shoot regeneration and genetic transformation of wild strawberry (*F. vesca*) (*9–14*). The shoot regeneration response of leaf tissue has mainly been related to the genotype and the cultivation factors, mainly in terms of the media composition (phytohormones and the type of nutrient medium). Cultivated strawberry varieties have shown large variabilities in the cell differentiation competence of their somatic tissues, and the effects of plant growth regulator (PGR) treatments in the induction of this process appear to be related to specific genetic factors (*6*).

The regeneration media that has generally produced the greatest shoot regeneration included the Murashige and Skoog (MS) medium (*15*) supplemented with 6-benzyladenine (BAP) and indole-3-butyric acid (IBA) (*6,16*). The ability of 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea [thidiazuron (TDZ)] to induce high shoot regeneration efficiency, in woody plant tissues in particular, has also been reported (*17–19*). The effect of TDZ in strawberry has been explored recently in a restricted number of *Fragaria* × *ananassa* cultivars. With this cytokine-like PGR shows specific responses that were dependent on the genotype and the type of tissue (*6*). Among the auxins, 3-benzo[b]selenienyl acetic acid (BSAA) is a new highly active molecule that has already been tested in some crops, where it has shown a highly effective activity for the induction of somatic embryogenesis (*20*). Although it has not yet been tested for its capability to control organogenesis in woody plants, we recently achieved interesting results—particularly in strawberry genotypes.

Among several strawberry genotypes tested in our trials the regeneration and transformation response varied mainly depending to the genotype. The highest efficiency in genetic transformation (about 5%) was observed from the genotypes with the highest leaf tissue regeneration efficiency (100% of leaf tissue regeneration), such as Sveva, a newly released octoploid cultivar (*F.* × *ananassa*) and the diploid *F. vesca* cv. Alpina W.O. Genotypes such as cv. Onda and cv. Paros, performing with an intermediate leaf tissue regeneration efficiency (about 80% of leaves with adventitious shoots) showed a reduced *Agrobacterium* transformation efficiency (1–3%). Strawberry genotypes showing much lower percentages of leaf tissue regeneration (lower then 40%) can be quite more difficult to transform.

The achievement of stable genetic transformation of plants is also strictly related to the antibiotic (kanamycin) selection protocol, starting from the early stage of leaf tissue regeneration—immediately after the *Agrobacterium* infection and co-culture—and including the in vitro proliferation and rooting of newly selected shoots.

Depending to the efficiency of the regeneration and transformation protocol, the first stable newly produced transgenic line can be available after 5 to 6 mo from the first transformation experiment.

2. Materials

2.1. Agrobacterium tumefaciens *Strains, Vector, and Plant Selectable Markers*

1. *A. tumefaciens* strains LB4404 and EHA105 are the most commonly used, and probably the most efficient, for strawberry genetic transformation.
2. The pBI121 binary plasmid was also successfully used (*21*).
3. The neomycin phosphotransferase II (*npt*II) gene is the selectable marker most commonly used and it is recommended for the highest efficiency in cleaning transgenic regenerants at the chimeric state and finally in selecting stable transgenic clones.

2.2. Explant Material

Newly expanded entire leaves, from in vitro proliferating shoots, cut transversally and cultured with the abaxial surface in contact with the regeneration medium.

2.3. Stock Solutions and Supplies

1. TDZ (Duchefa, Haarlem, The Netherlands) (1 mg/mL stock solution): To prepare, place 100 mg of TDZ in a tube with a magnetic stir bar, add 1 drop of dimethyl sulfoxide (DMSO) and 100 mL of deionized sterile water. Allow TDZ to dissolve by stirring. Store in a bottle and store at –20°C for up to 6 mo.
2. BSAA (Sigma) (1 mg/mL stock solution): To prepare, place 100 mg of BSAA in a tube with a magnetic stir bar, add 100 mL of deionized sterile water. Allow BSAA to dissolve by stirring. Place in a bottle and store at –20°C for up to 6 mo.
4. Rifampycin (33.3 mg/mL stock solution) (Duchefa): To prepare, place 66.6 mg/mL rifampycin in a baker with a magnetic stir bar and 2 mL of DMSO. Allow rifampycin to dissolve by stirring. Filter-sterilize and divide into 1-mL Eppendorf tubes. Store at –20°C for up to 6 mo.
5. Streptomycin (100 mg/mL stock solution) (Duchefa): To prepare, place 200 mg streptomycin in a baker with a magnetic stir bar and 2 mL of deionized sterile water. Allow streptomycin to dissolve by stirring. Filter-sterilize and divide into 1-mL Eppendorf tubes. Store at –20°C for up to 6 mo.

6. Kanamycin monosulfate (50 mg/mL stock solution) (Duchefa): To prepare, place 200 mg kanamycin monosulfate in a baker with a magnetic stir bar and 4 mL of deionized sterile water. Allow kanamycin to dissolve by stirring. Filter-sterilize and divide into 1-mL Eppendorf tubes. Store at −20°C for up to 6 mo. This substrate can be prepared and stored at 4°C for not more of 2 wk.
7. Cefotaxime (100 mg/mL stock solution) (Zariviz-Aventis): The commercial product consists of 1 g of powder that has to be dissolved in 10 mL of deionized sterile water, and mixed vigorously together. Cefotaxime can then be stored at 4°C for up to 6 mo. The antibiotics are added to the culture media after sterilization, as they are heat-sensitive.
8. Acetosyringone (20 mg/mL stock solution): To prepare, place 100 mg of acetosyringone in a baker with a magnetic stir bar, add 5 mL of ethanol absolute (Sigma), allow acetosyringone to dissolve by stirring. Store at −20°C for up to 6 mo.
9. Proline (200 mg/mL stock solution): To prepare, place 1 g of proline in a baker with a magnetic stir bar and 5 mL of deionized sterile water. Allow proline dissolve by stirring and store at −20°C for up to 6 mo.
10. Soil mix for plant acclimatazion: It is a mixture of commercial peat (neutral pH) and agriperlite (1:1). The mixture is autoclaved at 121°C and 1 bar pressure for 40 min before using.

2.4. Media

After pH adjustment, all media should be autoclaved at the temperature of 121°C and 1 bar pressure for 20′.

1. Shoot proliferating medium: 4.3 g/L of MS micro and macroelements (Duchefa), 5 mg/L of piridoxine, 5 mg/L of nicotinic acid, 20 mg/L of glycine, 100 mg/L of *myo*-inositol, 10 mg/L of thiamine, 30 g/L of sucrose, 0.5 mg/L BA (N^6-benzyl adenine (Sigma), and 7.5 g/L of agar, pH 5.7. This medium can stand at room temperature for 2 wk.
2. YEB medium for *Agrobacterium*: 1 g/L of yeast extract (Sigma), 5 g/L of beef extract (Sigma), 5 g/L of peptone (Sigma), 5 g/L of sucrose, 2 m*M* $MgSO_4$, 50 mg/L of kanamycin, 100 mg/L of rifampycin, 100 mg/L of streptomycin, and 7.2 g/L of agar (for solid media), pH 7.8. This medium can be stored at 4°C for no more than 2 wk.
3. Leaf infection solution: 4.3 g/L of MS micro and macroelements, 5 mg/L of piridoxine, 5 mg/L of nicotinic acid, 20 mg/L of glycine, 100 mg/L of *myo*-inositol, 10 mg/L of thiamine, and 20 g/L of sucrose, pH 5.2, 1 µL/mL of proline (200 mg/mL stock solution) and 1 µL/mL of acetosyringone (20 mg/mL stock solution). These last two components are added after sterilization, as they are heat-sensitive. The infection solution should be prepared fresh as required.
4. *Agrobacterium*-plant co-culturing medium: 4.3 g/L of MS micro and macroelements, 5 mg/L of piridoxine, 5 mg/L of nicotinic acid, 20 mg/L of glycine, 100 mg/L of *myo*-inositol, 10 mg/L of thiamine, 30 g/L of sucrose, pH 5.7, 1 µL/mL of proline (200 mg/mL stock solution), 1 µL/mL of acetosyringone (20 mg/mL

stock solution), and 2.65 g/L phytagel. This substrate can be prepared and stored at 4°C for no longer than 2 wk.

5. Leaf tissue washing solution: Sterile H_2O added with 500 mg/L cefotaxime. Prepare fresh as required.
6. Leaf explants regeneration and selection medium: 4.3 g/L of MS micro and macroelements, 5 mg/L of piridoxine, 5 mg/L of nicotinic acid, 20 mg/L of glycine, 100 mg/L of *myo*-inositol, 10 mg/L of thiamine, 30 g/L of sucrose, pH 5.7, 1 mg/L of TDZ, 0.2 mg/L of BSAA, 2.65 g/L phytagel, 200 mg/L of cefotaxime, and 25 mg/L of kanamycin monosulfate (50 mg/mL stock solution).
7. Rooting medium: 4.3 g/L of MS micro and macroelements, 5 mg/L of piridoxine, 5 mg/L of nicotinic acid, 20 mg/L of glycine, 100 mg/L of *myo*-inositol, 10 mg/L of thiamine, 30 g/L of sucrose, pH 5.7, 7.5 g/L of agar, 0.5 mg/L of IBA, 200 mg/L of cefotaxime, and 25 mg/L of kanamycin monosulfate.

3. Methods

The protocol includes three main steps: (1) the supply of plant material (in vitro proliferating shoots), (2) the *Agrobacterium* infection, and (3) the regeneration and selection of stable transformed 'plantlets'.

3.1. Preparation of Strawberry Leaf Tissue

1. For each genotype start in vitro proliferating shoots by sterilizing lateral buds collected from runners (vegetative parts of the mother plant), and treating them with 2% (v/v) chloride-active solution for 20 min.
2. After rinsing 4 to 5 times with sterile distilled water, transfer the meristematic tissues to sterile glass tubes (11-cm length) (Sigma) containing the shoot proliferating medium, to let meristematic tissues develop and generate shoots.
3. Incubate the proliferating shoots in growth chambers for 16-h photo period at 250 $\mu E/m^2/s$ and 8-h dark at 25°C. Subculture regularly at 4-wk intervals.
4. The transformation and regeneration experiments are carried out by using young expanded leaves detached from 4-wk-old in vitro proliferating shoots, after a minimum of 4 to 5 subcultures from the initial explants. When detached, incise the leaf laminar on transversal nervature and keep in sterile distilled water until starting the transformation experiment (*see* **Notes 1–3**).

3.2. Agrobacterium tumefaciens *Preparation and Plant Tissues Infection*

1. *Agrobacterium tumefaciens* strain is grown in YEB solid medium containing kanamycin, rifampycin and streptomycin in 90-mm diameter Petri dish, cultured at 4°C and transferred every 1 to 2 mo to a new YEB solid medium.
2. *Agrobacterium* inoculation suspension can be prepared by using YEB liquid medium added with the selecting (50 mg/L kanamycin) and cleaning antibiotics (100 mg/L rifampycin and 100 mg/L streptomycin).
3. Inoculate a small amount (one full handle) of *Agrobacterium* colonies from YEB solid medium to a 50-mL Falcon tube containing 10 mL of YEB liquid medium

supplemented with 50 mg/L kanamycin, 100 mg/L rifampycin, and 100 mg/L streptomycin.

4. Seal the tubes with parafilm and incubate the culture overnight at 29°C on a shaker (100–150 rpm).
5. After 24 h, take 1 mL of the *Agrobacterium* suspension and inoculate 9 mL of YEB liquid medium (with antibiotics) in a new 50-mL falcon sterile tube. Put the tube in the culture-conditions as described above.
6. On the day of transformation experiment, quantify bacteria growth at the spectrophotometer. Take 100 µL of bacteria culture and 900 µL of YEB liquid medium and put in a 1-mL couvette. The desired optical density (OD) value is approx 0.8 at 600 nm, corresponding to an inoculum density of 10^8 bacterial cells/mL *(22)*.
7. To prepare the inoculum suspension with the desired cell density (OD_{600} = 0.8) centrifugate bacteria suspension culture at 1600*g* for 15 min, discard the supernatant, and re-suspend the pellet in the infection solution. Add an appropriate volume of infection solution to the pellet (about 100 mL for each one OD value) so to have a total final re-suspension solution of 50 mL, which is normally sufficient for infecting between 100 and 300 leaves.
8. The infection medium is supplemented with 1 µL/mL (1 m*M*) both of proline and acetosyringone to induce the virulence (*vir*) genes of *Agrobacterium*, so when prepared close the tubes with parafilm and mix vigorously, then put the tubes at 28°C on a shaker (100–150 rpm), for 5 h, so to activate *Agrobacterium* cell division and virulence.

3.3. Explant Tissue Infection

1. Infection and co-cultivation is the main step of leaf tissue transformation. When the detached and cut leaves are ready and maintained in sterile H_2O to prevent drying, then gently wash the leaves in 30 mL infection solution containing the *Agrobacterium tumefaciens* strain for 15 min at 100 to 150 rpm.
2. After infection the leaves are blotted with sterile filter paper and transferred to the *Agrobacterium*-plant co-culturing medium for 48 h at 25°C in dark conditions. It is necessary to put the leaves with their inferior-adaxial side in contact with the medium.
3. At the end of the co-culturing period stop the infection by transferring the leaves to the washing solution for 5 h at 25°C with shaking at 100 to 150 rpm.

3.4. Leaf Tissue Regeneration and Selection

1. The washed leaves are then blotted with sterile filter paper and placed on leaf explants regeneration and selection medium, always by keeping in contact with the medium the adaxial side of the leaf.
2. For strawberry, the highest leaf tissues regeneration response is promoted by a first period of incubation (2 wk) in continuous dark followed by 16-h at 250 µE/m^2/s and 8-h dark, at 25°C. Leaf tissue has to be sub-cultured regularly at 4-wk intervals on freshly prepared media.
3. At each subculture, the type of morphogenic activity occurred for each explant should be monitored. Generally, yellow pale callus will form at the cut leaf edge

around the end of the first subculture. After transfer to the 16-h light/8-h dark photoperiod incubation condition, green dot-nodules start to form from the growing calli, whereas the remaining parts of the leaves display progressive necrosis.

4. The leaves bearing callus and green-dot nodules are subcultured every 3 wk on a freshly prepared regeneration and selection medium. Keep subculturing the leaf and callus tissues as long as it remains a proliferation and differentiation activity (up to 6–8 mo).
5. When transplanting, detach from the callus tissues the selectable green regenerated shoots (about 2–4 cm in size) and transfer them in glass tube (12-mm diameter) containing the shoot proliferation medium supplemented with the selection (kanamycin 25 mg/L) and decontaminant (cefatoxime 200 mg/L) antibiotics used in the regeneration medium. A larger number of isolable regenerants generally occurs at the second and third subculture (*see* **Note 4**).
6. The proliferation stage of the isolated shoots is another critical stage for the identification of stable homogenous transgenic lines. Again, 2 to 3 subcultures on the same proliferation medium supplemented with antibiotics are generally useful to identify the regenerated lines showing the more homogenous green tissues (*see* **Note 5**).

3.5. Rooting and Transplanting to Soil

1. Transfer the green stable proliferating lines on a rooting medium. A first proof of new stable putative transgenic lines is their ability to root in the presence of kanamycin. Usually plantlets start rooting after 3 wk. Only regenerated shoots that are able to root on medium with kanamycin are considered new putative transgenic plants. A polymerase chain reaction (PCR) analysis can be expected for a first molecular confirmation of the transgenic event.
2. Rooted shoots are transplanted into small pots, containing the soil mix for plant acclimatazion, and covered by a transparent cap allowing light pass through. Start the acclimatization of at least 10 to 15 micropropagated shoots for each new selected regenerants. During the acclimatization phase, the rooted shoots need to be irrigated every day in order to prevent drying. This first step of acclimatization is generally critical and only the accurate watering management can prevent shoot mortality.
3. After 3 to 4 wk the caps are removed and after 1 additional wk plants can be transplanted into bigger plots and placed in a standard greenhouse, carring an upper shadow to prevent direct exposition to sun light. Generally, start the molecular characterization of the transgenic events at this stage (Southern blot).
4. Plants can be grown in a standard greenhouse, and vegetatively propagated by runners so as to produce the material useful for starting the risks and benefits assessment at open field conditions as requested for transgenic plants (*see* **Note 6**).
5. Transgenic clones are cultured in a greenhouse and/or transferred to open field trials, depending on the agronomic interest and with respect of the rules for genetically modified organism (GMO) experimental trials. At each cultivation cycle and for each clone, new plants are produced by vegetative propagation (runners).

4. Notes

1. Experiment planning: Each experiment has to be scheduled keeping in mind the *Agrobacterium* infection preparation as well as the proliferating shoots as sources of the leaf tissues.
2. Experiment repetition: Plan at least three subsequent transformation experiments, with at least 50 or 100 leaf explants each, depending on the efficiency of the regeneration protocol.
3. It is of extreme importance to ensure sterile conditions for the entire experiment process.
4. Each newly selected regenerant has to be identified with the corresponding leaf tissue origin. This leaf tissue can be maintained for other subsequent subcultures; if other regenerating shoots occur later they can be isolated, but it is better to identify them as a sub-clone of the first regenerated shoots already isolated from the same explants. If both will grow stably on proliferation–selection medium, only the molecular characterization (Southern blot) can confirm their origin from different transformation events. If the regeneration and transformation protocol is really efficient, then as soon as a regenerated shoot is isolated the leaf the explant can be discarded.
5. The identification and selection of more green stably proliferating and rooting shoots is a fundamental aspect of the in vitro procedure to avoid the risk of selecting chimeric clones.
6. To have a first molecular proof of the transformation event a PCR analyses can be performed on isolated lines able to root on kanamycin. Southern blot molecular characterization can be performed on plant tissue from acclimatized plants.

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Walnut (*Juglans*)

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Summary

Walnut species are important nut and timber producers in temperate regions of Europe, Asia, South America, and North America. Trees can be impacted by *Phytophthora*, crown gall, nematodes, and cherry leaf roll virus; nuts can be severely damaged by codling moth and *Xanthomonas* blight. The long-generation time of walnuts and an absence of identified natural resistance for most of these problems suggest biotechnological approaches to crop improvement. Described here is a somatic embryo based transformation protocol that has been used to successfully insert horticulturally useful traits into walnut. Selection is based on the combined use of the selectable neomycin phosphotransferase (*npt*II) gene and the scorable *uid*A gene. Transformed embryos can be germinated or micropropagated and rooted for plant production. The method described has been used to establish field trials of mature trees.

Key Words: *Juglans regia*; *Juglans hindsii*; *Juglans nigra*; Persian walnut; California black walnut; Eastern black walnut; Paradox; gene transfer; somatic embryo; *Agrobacterium tumefaciens.*

1. Introduction

There are approx 20 species of walnut worldwide, many of which are valued for their nut production and timber quality. The principle species used for commercial walnut production, the Persian walnut (*Juglans regia* L.), is native to the mountain ranges of central Asia. It is now cultivated in many temperate regions including much of the Mediterranean, Central Asia, northern India, China, South Africa, Argentina, Chile, and Australia. In the United States this crop is produced almost entirely in California, where orchard trees are grafted onto rootstock of either the native California black walnut *(Juglans hindsii*) or *J. hindsii* × *J. regia* hybrids known as Paradox. The Eastern black walnut

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(*Juglans nigra*), native to the eastern United States, is highly valued for its timber quality and has been introduced into Europe and China for this purpose.

Walnuts are susceptible to a number of insect, disease, and nematode problems. The key insect pest is codling moth (*Cydia pomonella* L.), and *Xanthomonas* bacterial blight can cause substantial nut loss. Cherry leafroll virus (blackline disease) is pollen disseminated and can kill grafted trees at full production. Rootstock diseases seriously impacting both nurseries and growers include crown gall disease (*Agrobacterium tumefaciens*) and *Phytophthora* crown and root rots. Nematodes are increasingly serious pests in nurseries and in orchard establishment because of the loss of available fumigants. Genetic resistance for most of these problems has not clearly been identified in walnut and walnut breeding is a very lengthy process. Embryo rescue (*1*) and *Agrobacterium*-mediated transformation have both been used in attempting to address these pest problems.

Walnut hybrids can be micropropagated for rootstock production (*2*), or Persian walnut cultivars can be produced on their own roots (*3*) to avoid graft costs and blackline disease. Repetitively embryogenic somatic embryo cultures can be produced easily from immature zygotic embryos (*4*) and with difficulty from immature catkins (unpublished data). Triploid walnuts have been produced from endosperm (*5*). *Agrobacterium*-mediated transformation of walnut somatic embryos was first reported using marker genes (*6,7*). Subsequent work showed these methods could be applied to generate transgenic walnuts expressing a modified *Bacillus thuringiensis* gene for insect resistance (*8,9*), the *rol*ABC genes from *A. rhizogenes* for short internodes and altered root architecture (*10*), and RNAi constructs to inhibit crown gall formation (*11*). A number of additional genes that were successfully transformed into and expressed in walnut did not produce useful phenotypes including the LFY gene from Arabidopsis, the GNA snow drop lectin gene, and the Xa21 *Xanthomonas* resistance gene from rice (McGranahan and Dandekar, unpublished data).

The protocol detailed here is based on our experience using repetitively embryogenic walnut somatic embryo cultures. New somatic embryos develop from single epidermal cells on existing embryos (*12*). This process automatically eliminates any chimeras so nonchimeral transformants can be selected by picking from second generation embryos. We used both the neomycin phosphotransferase (*npt*II) as a selectable marker and the *uid*A gene as a scorable marker. Nontransformed embryos multiply poorly and generally develop bad form and a yellowish color on kanamycin-containing medium. Embryos which multiply well and appear healthy on kanamycin can be checked for β-glucuronidase (GUS) activity using X-glucuronidase staining (*13*) or green fluorescent protein (GFP) fluorescence (*14*). Transformed embryos can be germinated following desiccation (*15*). This method has also been used to transform Eastern Black Walnut (*16*) and pecan (*17*).

Transformation efficiency (the percent of initially treated embryos [E_0] that produce one or more non-chimeric transformed second generation [E_2] embryos which continue to be embryogenic) is approx 20 to 25% *(**7,9**)*. Bacterial overgrowth can sometimes be a problem and embryo germination rates are relatively low. The latter may be circumvented by micropropagating transformed epicotyls and either rooting the resulting microshoots or budding them to seedling rootstocks *(**18**)*.

2. Materials

2.1. Plant Material

Immature walnuts of the variety or species of interest, preferably harvested 6 to 10 wk post anthesis, or previously established walnut somatic embryo cultures.

2.2. Transformation Vectors and A. tumefaciens *Strains*

1. *A. tumefaciens* strains that work efficiently for walnut are the disarmed derivatives like EHA101 *(**19,20**)* of the tumorigenic A281 strain that harbors the Ti plasmid pTIBo542 and the nonpathogenic strain C58C1 *(**20,21**)* which contains a disarmed version of the tumorigenic Ti plasmid pTiC58 (*see* **Note 1**).
2. The binary system for walnut transformation is completed with the introduction of broad host range binary plasmids that contained the desired T-DNA region. For this we use derivatives of binary plasmids described by McBride and Summerfelt *(**22**)*. These derivatives have been exclusively used for the *Agrobacterium*-mediated transformation protocol described here. This binary contains the selectable marker gene APH(3)II for kanamycin resistance and has been modified to contain the scorable marker gene *uid*A encoding GUS (*see* **Note 1**). Binary vectors can be introduced into *Agrobacterium* strains by a number of methods as described in **Chapter 3**, Volume 1 (*see* **Note 2**).

2.3. Stock Solutions

1. 100 mL Kanamycin sulfate (50 mg/mL): Dissolve 5 g kanamycin sulfate powder in 100 mL of water, filter-sterilize, and freeze in 10 to 15-mL aliquots in 25-mL screw cap vials.
2. 10 mL Gentamycin sulfate (25 mg/mL): Dissolve 250 mg gentamycin sulfate powder in 10 mL of water, filter-sterilize, and store frozen.
3. 100 mL Cefotaxime (100 mg/mL): Dissolve 10 g of cefotaxime powder in 100 mL of water, filter-sterilize, and freeze in 10 to 15-mL aliquots in 25-mL screw cap vials.
4. Acetosyringone: Dissolve acetosyringone (3,5-dimethoxy-4′-hydroxyacetophenone) in ethanol to make a 100 m*M* (19.6 mg/mL) solution. Do this in a capped centrifuge tube and vortex so the ethanol does not evaporate. Seal the top well with Parafilm or plastic wrap to prevent evaporation and store at room temperature.
5. Proline: 100 mg/mL stock solution. Dissolve the powder in water, filter-sterilize, and store at 4°C.

6. X-Gluc staining solution: Dissolve X-gluc (5-bromo-4-chloro-3-indoyl glucuronide) in dimethylformamide to make a 0.3% wt/vol solution. Dilute with 100 m*M* sodium phosphate buffer, pH 7.0 containing 0.006% Triton X-100 and 0.5 m*M* K+Fe cyanide to make a 1 m*M* X-gluc working solution. Filter-sterilize and store refrigerated. Keeps for at least 1 yr.
7. Indole-3-butyric acid (IBA): Dissolve IBA potassium salt (K-IBA) in water to give a 0.1 mg/mL stock solution.
8. 6-Benzylaminopurine (BAP): Dissolve BAP powder in a few drops of 1 *N* KOH and dilute to volume with water to give a 1 mg/mL BAP stock solution.

2.4. Media

1. Driver Kuniyuki walnut (DKW) basal medium (Sigma; cat. no. D6162): Dissolve DKW powder in 30 g/L sucrose in water, dilute to volume, adjust pH to 5.5, add 2.1 g/L Gelrite® (Merck), Phytagel® (Sigma), or other brand of gellan gum to solidify, autoclave, and pour in 100 × 15-mm Petri plates.
2. *Agrobacterium* liquid growth medium: Use either 523 (*23*) medium or Luria–Bertani (LB) (*24*) medium:
   a. 523 Medium: 10 g/L sucrose, 8 g/L casein hydrolysate, 4 g/L yeast extract, 2.0 g/L $K_2HPO_4 \cdot 3H_2O$, and 0.15 g/L MgSO4 in distilled water. Adjust pH to 7.1 and autoclave.
   b. LB medium: 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl in distilled water. Adjust pH to between 6.8 and 7.2 and autoclave.
3. *Agrobacterium* growth plates (523 or LB): Prepare medium as described above adding appropriate antibiotics for the vector used and 15 g/L Bacto agar to solidify. Autoclave and pour in 100 × 15-mm Petri plates.
4. Virulence induction medium (IM): Prepare 100 mL or more liquid Murashige and Skoog (MS) basal medium (Sigma M5519) containing 20 g/L sucrose, 100 µ*M* acetosyringone, and 1 m*M* proline. Adjust pH to 5.2 and filter-sterilize. Store refrigerated in sterile 50-mL capped centrifuge tubes.
5. Acetosyringone medium (AS) plates: Prepare DKW basal medium containing 30 g/L sucrose and 100 µ*M* acetosyringone. Adjust pH to 5.5, add 2.1 g/L Gelrite, autoclave, and pour in 100 × 15-mm Petri plates (*see* **Note 3**).
6. KAN/CEF selection medium: Prepare basal DKW with 30 g/L sucrose, adjust pH to 5.5, dispense into 1-L screw-cap bottles (500 mL/bottle), add 1.05 g Gelrite® to each bottle, autoclave, and cool to 60°C in a water bath. Then add 200 mg/L pH adjusted (*see* **Note 4**), filter-sterilized kanamycin and 500 mg/L filter-sterilized cefotaxime (*see* **Note 5**). Mix thoroughly and pour into sterile 100 × 15-mm Petri plates. When solidified, store refrigerated in the original plastic sleeves until ready for use.
7. KAN only selection medium: The same medium as KAN/CEF selection medium but without the cefotaxime.
8. DKW shoot medium (Sigma D8785): Dissolve DKW shoot medium powder and 30 g/L sucrose in water, dilute to volume, adjust pH to 5.5, and add 2.1 g/L Gelrite® (Merck), Phytagel® (Sigma), or other brand of gellan gum to solidify. Microwave until the medium boils, mix thoroughly on a stir plate, dispense into Magenta

Corporation GA7 vessels (approx 30 mL of medium each), and autoclave. Alternatively, use DKW basal medium and add 1 mg/L BAP and .01 mg/L IBA.

2.5. Other Supplies and Chemicals

1. Sterile empty 100 × 15-mm Petri plates.
2. Sterile disposable 50-mL screw-cap centrifuge tubes.
3. Sterile disposable cotton-plugged 10 mL pipet.
4. Pipettors with sterile 1-mL and 200-μL tips.
5. Sterile disposable 6-well Multiwell plates.
6. Sterile disposable 96-well Multiwell plates.
7. Filter paper or paper toweling discs cut to fit in 100 × 15-mm Petri plates and autoclaved.
8. Filter paper or paper toweling discs cut to the diameter of the wells of a 6-well plate and autoclaved.
9. 150-mm diameter desiccator (Nalgene; cat. no. 5315–0150).
10. Saturated $ZnSO_4$ or NH_4NO_3 solution.
11. Driver Kuniyuki walnut micropropagation medium (Sigma; cat. no. D8785).
12. Magenta GA-7 vessels (Magenta Corp., Chicago, IL).
13. Ray Leach Cone-tainer SC-10 Super Cells (Hummert Corp).

3. Methods

3.1. Initiate or Obtain Actively Multiplying Walnut Somatic Embryo Cultures

1. Surface-sterilize immature intact walnuts in 15% Clorox® (6% sodium hypochlorite) for 10 to 15 min and rinse in sterile water.
2. Remove the zygotic embryos from the walnuts. To do this, hold the nut with the blossom end up. Cut 1 or 2 mm into the issue with a scalpel several mm above the midpoint (equator) and continue this cut all the way around the nut. Twist the blade slightly to flip the blossom end off. The embryo will be exposed in the center and can be excised with a scalpel.
3. Culture zygotic embryos on basal DKW medium. Culture 1 to 5 embryos per plate, depending on the embryo size. Leave 1 to 2 cm of space between embryos to facilitate rescue of clean embryos if one is contaminated during excision. Place the embryos on the surface, not in the medium. The orientation is not critical, but once established transfer embryos in the same orientation so the same surface is always on the medium. Place in the dark at room temperature.
4. Transfer to fresh medium weekly until somatic embryogenesis is observed. Continue to transfer every 1 to 2 wk, allowing embryos to multiply until enough material is available to proceed.

3.2. Agrobacterium *Preparation*

1. Streak *Agrobacterium* from glycerol stock onto a 523 or LB plate with appropriate antibiotics for the vector used. Incubate at 28 to 30°C for 2 d or until good bacterial growth occurs. Then store refrigerated if a longer time period is required before use.

2. For each construct to be used, inoculate liquid cultures (one or two 50-mL conical tubes with approx 20 mL each liquid 523 or LB medium) with a loop of bacteria from the plates and place the capped tubes on a rotary shaker at moderate speed (200 rpm) at room temperature (about 25°C).
3. After approx 2 h, add the appropriate selective antibiotics for the vector used and return to shaker.
4. After shaking overnight the bacterial cultures should be turbid. Determine the A_{600} by reading a 1:10 diluted sample in a spectrophotometer.
5. Calculate the amount of *Agrobacterium* culture to use to obtain the desired volume of co-cultivation suspension at the desired bacterial concentration (an A_{600} reading of 0.5 is equivalent to 2.5×10^{-8} bacteria/mL) using the following formula:

$$\frac{(\text{Amount of suspension wanted}) \times (A_{600} \text{ of desired conc.})}{(A_{600} \text{ reading you get}) \times \text{dilution factor}}$$

Example: You want to make 25 mL of co-cultivation solution at a concentration of 2.5×10^{-8} bacteria/mL. You used a 1:10 dilution in the spectrophotometer and got a reading of 0.371. How much of the original culture do you need?

$$\frac{(25\text{ mL}) \times (0.5)}{(0.371) \times 10} = 3.36\text{ mL}$$

6. Using a sterile pipet, place the calculated volume of culture solution into a sterile plastic capped 50-mL centrifuge tube and centrifuge sufficiently to lightly pellet the bacteria (e.g., 10 min at 4000*g*).
7. Pour or pipet the supernatant into an autoclaveable waste container and resuspend the pellet in the co-cultivation medium. The pellet is easier to resuspend in a small volume (0.5 mL first) using a pipet tip. Then add the full volume. If needed, set up a tube for a no bacteria control using only co-cultivation medium.
8. Return the tubes to the shaker until ready to use.

3.3. Co-cultivation

1. Based on the number of genotypes to be transformed and the number of vectors employed, determine the number of total treatments.
2. Put sterile filter paper or paper towel discs in the bottom of the appropriate number of wells in 6-well Multiwell plates. This will make it easier to remove small embryos after co-cultivation.
3. Select actively growing somatic embryos of the genotypes to be used and for each vector to be used with that genotype, fill a well $^1/_2$ to $^2/_3$ full of embryos (*see* **Note 6**).
4. Dispense the appropriate *Agrobacterium* co-cultivation suspension into each well using sterile 10 mL pipettes with cotton plugged ends (*see* **Note 7**). Allow to sit for at least 10 to 15 min or longer until ready for the next step (*see* **Note 8**).
5. Place sterile filter paper or paper towel discs in empty sterile Petri plates—one for each treatment—and label them.

6. Remove as much excess co-cultivation liquid as possible from each well to an autoclaveable waste container. Pipetting with a 1-mL sterile tip works well. Keep the tip against the side to avoid plugging with small embryos. To avoid any cross contamination from splashing, a sterile waste container—such as a Magenta container or small sterile jar—for each construct is useful.
7. Transfer the embryos from the wells to the Petri plates. The larger embryos can be picked up with sterile forceps. Then carefully pick up the filter paper in the well with two forceps and set the whole thing on the dry paper in the Petri plate. This blots off additional excess liquid.
8. Transfer the embryos (about 10/plate—keep them well spread out) to plates of AS medium and place in the dark for 48 h at 20 to 22°C.

3.4. Selection on Kanamycin

1. After co-cultivating for 48 h, transfer the embryos to plates of KAN/CEF selection medium (*see* **Note 9**) containing 200 mg/L kanamycin and 500 mg/L cefotaxime (*see* **Note 10**). Incubate the culture plates in the dark at room temperature.
2. Transfer embryos to fresh KAN/CEF medium after another 48 h and again after the first wk. This helps to reduce bacterial overgrowth. Then transfer weekly for 8 to 12 wk.
3. As new somatic embryos begin to emerge, separate them from the parent (E_0) embryos. Label these as E_1 embryos. Repeat this process for one more generation (E_2 embryos).
4. After 6 to 8 wk of selection, embryos can be moved to selection medium containing only the kanamycin. Removing the cefotaxime at this point ensures that the embryos no longer have any residual *Agrobacterium* and avoids unnecessary expense for cefotaxime.

3.5. Scoring for GUS Expression

1. As E_2 embryos emerge test them for GUS (*uid*A) activity.
2. Pipet 40 µL of X-gluc working solution into wells of a sterile 96-well Multiwell plate.
3. Using a fine point scalpel or by twisting off with a pair of forceps, remove a small piece of tissue (cotyledon tips work well) from each well formed and healthy E_2 embryo of interest. Put the tissue piece in the X-gluc and label and mark the location of the embryo from which it was excised.
4. Watch for blue color. Color change should be apparent in 10 min to 2 h.
5. If tissue turns blue, propagate more somatic embryos from the tested E_2 embryo (*see* **Note 11**).

3.6. Germination and Plant Production

1. After sufficient additional somatic embryos have developed, desiccate some to initiate germination. Choose well formed somatic embryos and place them in 35 × 10-mm sterile Petri plates with no medium. Cover the plates but leave unsealed (do not wrap with Parafilm) and place them in the dark at room temperature on the

rack of a well sealed desiccator containing 10 to 15 mL of saturated $ZnSO_4$ or NH_4NO_3 in the bottom.

2. After 2 to 7 d, when the embryos become an opaque white with the consistency of popcorn but before they brown, remove the embryos from the desiccator and place them on DKW shoot medium in Magenta GA-7 vessels or glass jars with similar head space. Culture at room temperature under cool white fluorescent lights (16-h day photoperiod, approx 100 $\mu E/m^2/s$) for 2 to 8 wk.
3. Most embryos will produce roots, but typically fewer than 10% of embryos develop shoots. Roots will usually emerge from embryos in a week to 10 d. A few shoot buds may also begin to push quickly. In this case, the plants should be removed from the medium as soon as possible and planted in potting soil. If the embryos are left on the shoot medium the roots begin to deteriorate but more shoot buds will push. These can be excised and micropropagated. Alternatively embryos can be placed on shoot medium for 1 wk to initiate germination and then transferred to DKW basal medium. This will not push as many shoots but will give more intact plants.
4. Embryos that develop both shoots and roots can be transplanted to any well drained potting soil, for example UC Mix (25:42:33% sand:fir bark:peat moss). Plant in a container that drains well, for example, SuperCell ($8^1/_2 \times 1^1/_2$) containers from Hummert. Plastic cups with holes punched in the bottoms work for small numbers.
5. To acclimatize, keep plants at 100% humidity for 2 wk and then gradually reduce the humidity. Covering small pots with plastic bags works well for small numbers. Then gradually open the bags over a 2-wk period by creating small holes and then enlarging them, until the plants are fully acclimated. For larger numbers, place potted plants in a fog (not mist) chamber for 2 wk and then keep moist (fog if possible) on an open bench and reduce humidity gradually over the next 2 wk. Water and fertilize daily with half strength Hoagland's solution, Miracle Gro®, or other commercially available complete fertilizer. Keep plants under 16-h photoperiod at 25 to 28°C. If under artificial light, provide as much light as possible. If in the greenhouse, whitewash the glass or provide shade cloth during the summer.
6. Established plants can be repotted to larger containers as needed and maintained in a greenhouse or lath house.

4. Notes

1. Walnut is susceptible to wide variety of *Agrobacterium* strains. This was discussed in an earlier publication *(22)* where we showed the susceptibility of walnut vegetative tissues to a variety of strains. Among the strains that are particularly infective are the derivatives of A281, A6, and C58. We have used mainly the disarmed versions of A281 and C58 in all of our transformation experiments. With the exception of apical meristems we found most vegetative tissues quite susceptible to the infection with *Agrobacterium* including somatic embryos; the latter was noted in our earlier publication *(6)*. The *npt*II gene works when higher concentrations of kanamycin are used; typically 100 μg and above. Lower concentrations do

not work particularly well. Because of the weak selection with kanamycin, and the variability in the efficiency of transformation this may produce, we used GUS to confirm the transformants. Among the GUS constructs the gene that contains an intron has the least background, but the others work as well too.

2. We use electroporation to introduce DNA into *Agrobacterium*. Briefly, 1 µL of plasmid DNA (10–100 ng) is added to *Agrobacterium* competent cells. The mixture is placed on ice for 2 min before being transferred to a pre-cooled 0.2-cm electroporation cuvet (BioRad). Apply voltage with settings at 2.5 kv (field strength), 25 µF (capacitance), 400 Ω (resistance) at time constants of 8 to 12 m/s. One milliliter of YEP rich media is added to the eletroporated cell/DNA mixture. Incubate it with shaking at room temperature for 45 min and then plate on selective media.
3. The 100 m*M* acetosyringone stock should be at room temperature. Be sure it is in suspension. If it has been cold you may need to warm it in warm water and vortex briefly to re-suspend. To make 100 µ*M* acetosyringone medium (AS medium) add this stock to DKW basal medium before autoclaving.
4. Kanamycin sulfate in solution has a very high pH. If used at a concentration greater than 100 mg/L for selection, the kanamycin begins to raise the pH of the medium, altering the salt solubility and gelling properties. For this reason, one may prefer to adjust the pH of the kanamycin stock solution to 5.5. To do so, dissolve the kanamycin powder in water to about half the desired final volume, adjust to pH 5.5 using 1 *N* HCL, and dilute with water to final volume.
5. Both cefotaxime and carbenicillin were tried initially. Carbenicillin showed an auxin like effect that reduced embryo quality and its use was abandoned. Cefotaxime actually improves both the quality and the multiplication rate of non-transformed embryos but is not used for routine culture because of expense. We have not tried other antibiotics possibly suitable for eliminating the *Agrobacterium* (e.g., timentin).
6. It is helpful to pick out embryos to use ahead of time and place them on plates of DKW basal medium. This will give you an idea of how many you will have to work with before you get too far into the rest of the work. If you need more, wait another wk or lower your selection standards. To achieve the best transformation efficiency it is important to use rapidly multiplying embryos. Embryos multiply continuously and a culture will have a mixture of all sizes, ages, and qualities of embryos. If you want to measure transformation rates, timing, or otherwise need uniform initial material for experimental purpose, choose small, white (2–5 mm) embryos of good somatic embryo form (two visible cotyledons) and semi-translucent rather than ivory white opaque appearance. This gives starting material that is distinct, can be counted easily, and these embryos will continue to multiply well. Opaque white embryos often move towards germination and produce fewer new embryos. Once you have selected the embryos, distribute them equally across treatments. Mark one or more plates for each treatment and then select similar sets of embryos to be used for each treatment. Continue this process until all the embryos are assigned a treatment. Be very careful with your sterile technique

because you can contaminate everything during this process. If the goal is only to obtain some transformants, use any rapidly multiplying embryo culture material except browning older embryos.

7. Do this soon after placing embryos in the wells so they don't dry out. Use enough medium to cover the embryos (approx 8 mL/well) if you have enough; otherwise, distribute the medium over the embryos so they all get wet. Label carefully and be sure to avoid cross contamination. You may want to use a different plate for each vector.
8. Physical wounding is not necessary, and is, in fact, detrimental to successful transformation. Wound sites develop callus rather than new somatic embryos.
9. We tested the efficiency of applying kanamycin immediately after co-cultivation vs applying at a later stage. Early application gave a better yield of transformants.
10. Transfer embryos in a consistent pattern on each plate so that if resistant bacteria begin to multiply they are not moved to all the embryos on the plate.
11. The X-gluc is not always toxic. If you use a filter-sterilized X-gluc solution, dispense it into sterile wells, and return the tissue to selection medium as soon as the blue color becomes apparent, then tissue pieces that turn blue can sometimes themselves develop embryogenic cultures in addition to using the embryo from which it was excised.

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VI

Ornamental Plants

28

Carnation (*Dianthus caryophylus* L.)

Chalermsri Nontaswatsri and Seiichi Fukai

Summary

Carnation is a valuable crop for the cut flower industry and demand for new and improved varieties is growing. However, genetic transformation of carnations is currently limited because of a lack of efficient routine technique. In this chapter, we present an easy and effective protocol for gene transfer to carnation node explants and subsequent adventitious shoot regeneration. For high-adventitious shoot regeneration, node explants from first to third node of 5- to 8-cm long shoots were cultured on Murashige and Skoog (MS) medium, containing 1.0 mg/L thidiazuron (TDZ), 0.1 mg/L α-napthalenoacetic acid (NAA), 20 g/L sucrose, and 2 g/L Gellan gum for 10 d. Then the explants were cut into 8 radial segments and subcultured onto MS medium, containing 1.0 mg/L BA, 0.1 mg/L NAA, 20 g/L sucrose and 2 g/L Gellan Gum. For effective genetic transformation, 3- to 5-d precultured node explants were submerged in an *Agrobacerium* suspension for 10 min, then cocultivated on filter paper soaked with water and 50 μ*M* acetosyringone (AS). After cocultivation, the explants were cut into eight radial segments and subcultured onto selection medium until transformed shoots regenerated from the explants.

Key Words: *Agrobacterium*; genetic transformation; carnation (*Dianthus caryophylus* L.); adventitious shoot regeneration; node explants; gus (β-glucuronidase); *npt*II (neomycin phosphotransferase); AS (acetosyringone).

1. Introduction

One of the most serious problems with genetic transformation of carnation is low-transformation efficiency. There have been some reports of successful genetic transformation of carnation, but the protocols are still laboratory-dependent and far from routine. Therefore, development of an efficient, genotype-independent transformation method is crucial. Nevertheless, to establish a genetic transformation system, it is first necessary to develop an efficient shoot-regeneration method.

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Many factors affect successful genetic transformation, such as the strain of *Agrobacterium* used, plant condition, inoculation methods, and co-cultivation methods. Many reports indicate the importance of explant preculture by phytohormones for successful genetic transformation (*1–3*). Sangwan et al. (*2*) reported the dividing and shifting to primary cell wall structure of *Arabidopsis* cells after auxin/cytokinin treatment during the critical preculture period, can promote the passage of T-DNA into cytoplasm.

Co-cultivation is one of the most important steps for the genetic transformation of plants. Some researchers successfully transfer genes to explants cocultivated with a medium, rich with hormones, such as for *Cyclamen persicum* Mill. (*4*) and Tomato (*5*). Some have reported successful genetic transformation by reducing the concentration of the co-cultivation medium to half that of the regeneration medium (e.g., for *Drosera rotundifolia* L. [*6*]). Successful transformation of *Eustoma* has been achieved by using a low-nutrient co-cultivation medium (*7*). Studies have also indicated that acetosyringone (AS) is an important substance, which affects the success of genetic transformation (*8*). AS activates the virulence genes of *Agrobacterium* and enhances the transfer of foreign genes into plant genomes (*8*). In Loblolly Pine and Norway Spruce, Wenck et al. (*9*) found that AS was essential for high-transformation efficiency, but high concentrations (100 m*M* or greater) were not beneficial.

In the study reported here, we describe an easy and effective protocol for adventitious shoot regeneration and gene transfer to carnation node explants. In this protocol, a suitable cocultivation medium is important to increase transformation efficiency of carnation. Using this protocol, we have obtained transgenic carnation plants from five genotypes (Killer, Laurella, Master, Otome, and Tanga). The transformation efficiencies varies from 7 to 95% (β-glucoronidase (GUS) positive shoot/100 node explants infected) depending on genotypes (*10*).

2. Materials

2.1. Node Explants

Carnation 5 genotype (Killer, Laurella, Master, Otome, and Tanga) (*see* **Note 1**), 5- to 8-cm shoots, taken from green-house mother carnation plants, maintained at a minimum temperature of 15°C under natural day length.

2.2. Node Explants Culture Media and Stock Solutions

1. Thidiazuron (TDZ) stock (100 mg/L): dissolve 10 mg of TDZ in a few drops of 1 *N* NaOH and make up the volume to 100 mL with double distilled water (ddH_2O) and store at −20°C.
2. α-napthalene acetic acid (NAA) stock (100 mg/L): Dissolve 10 mg of NAA in a few drops of 1 *N* NaOH and make up the volume to 100 mL with ddH_2O and store at −20°C.

3. N^6-benzyladenine (BA) stock (100 mg/L): Dissolve 10 mg of BA in a few drops of 1 *N* NaOH and make up the volume to 100 mL with ddH_2O and store at −20°C.
4. Medium I: Murashige and Skoog (MS) medium (NH_4NO_3, 1650 mg/L, KNO_3, 1900 mg/L, $MgSO_4·7H_2O$, 370 mg/L, $CaCl_2·2H_2O$, 440 mg/L, KH_2PO_4, 170 mg/L, H_3BO_3, 6.2 mg/L, $MnSO_4·4H_2O$, 22.3 mg/L, $ZnSO_4·7H_2O$, 8.6 mg/L, $CoCl_2·6H_2O$, 0.025 mg/L, $CuSO_4·5H_2O$, 0.025 mg/L, KI, 0.83 mg/L, $Na_2MoO_4·2H_2O$, 0.25 mg/L, $FeSO_4·7H_2O$, 27.8 mg/L, $Na_2EDTA·2H_2O$, 37.3 mg/L, *myo*-inositol, 100 mg/L, thiamine HCl, 0.1 mg/L, pyridoxine HCl, 0.5 mg/L, nicotinic acid, 0.5 mg/L, and glycine, 2.0 mg/L) containing 1 mg/L TDZ , 0.1 mg/L NAA, and 20 g/L sucrose. Adjust pH to 5.75 using NaOH and add 2 g/L Gellan gum Wako (cat.no. 075-03075), and autoclave at 121°C for 15 min.
5. Medium II: MS medium containing 1 mg/L BA, 0.1 mg/L NAA, and 20 g/L sucrose. Adjust pH to 5.75 using NaOH and add 2 g/L Gellan gum, and autoclave at 121°C for 15 min.
6. Hyponex medium: 3 g/L Hyponex fertilizer (6.5:6:19) (Hyponex Co.Ltd.), 20 g/L sucrose, and 2 g/L Gellan gum. Autoclave at 121°C for 15 min.

2.3. Agrobacterium *Strain and Plasmid Vectors*

Agrobacterium tumefaciens, strain AGL0, having a binary plasmid pKT3 (***10***). This construct contains a screenable marker gene cassette with the nopaline synthase promoter (Pnos) driving the β-glucuronidase (*gus*) gene. The *gus* gene has an intron derived from caster bean catalase. The selectable marker gene cassette is the CaMV 35S promoter driving the neomycin phosphotransferase (*npt*II) gene.

2.4. Agrobacterium *Culture Medium and Co-cultivation Medium*

1. Solid Luria Bertani (LB) medium: 10 g/L tryptone, 5 g/L yeast extrat, 10 g/L sodium chloride, and 8 g/L agar, pH 7.0, supplemented with 50 mg/L kanamycin and 50 mg/L rifampicin. Autoclave and cool to between 50 and 60°C.
2. Liquid LB medium: 10 g/L tryptone, 5 g/L yeast extract, and10 g/L sodium chloride, pH 7.0. Autoclave and store at room temperature. Before using, suplement with 50 mg/L filter-sterilzed kanamycin and 50 mg/L filter-sterilzed rifampicin.
3. Co-cultivation medium: 50 µ*M* AS in sterilized water.

2.5. Antibiotics and Other Stock

All antibiotics are dissolved either in ddH_2O or dimethyl sulfoxide (DMSO), filter-sterilized, and stored in aliquots in −20°C. They should be added to various media after autoclaved and cooled down to around 55°C.

1. Kanamycin stock (50 mg/mL): dissolve 500 mg of kanamycin (Wako) in 10 mL of ddH_2O.
2. G418 (geneticin) stock (50 mg/mL): Dissolve 500 mg of G418 (Wako) in 10 mL of ddH_2O.
3. Vancomycin stock (200 mg/mL): Dissolve 1 g of vancomycin (Wako) in 5 mL of ddH_2O.

4. Cefotaxin stock (250 mg/mL): Dissolve 1 g of cefotaxin in 4 mL of ddH_2O.
5. Rifampicin stock (50 mg/mL): Dissolve in DMSO.
6. AS stock (19.62 mg/mL): Dissolve 196.2 mg of AS in 1 mL of ethanol and make up the volume to 10 mL with ddH_2O

2.6. Selection Medium

1. MS medium for selection: Medium II plus 20 mg/L of G418, 250 mg/L cefotaxin, and 400 mg/L of vancomycin.
2. Hyponex medium for selection: Hyponex medium plus 60 mg/L G418, 250 mg/L cefotaxin, and 400 mg/L vancomycin.

2.7. X-Gluc solution for GUS Assay (11)

1. X-Gluc solution: 0.5 m*M* 5-bromo-4-chloro-3 indolyl-β-D-glucuronide-cyclohexy (Wako), 0.5 m*M* Feri/Fero (Wako) dissolved in 100 m*M* phosphate buffer, pH 7.0, containing 20 % ethanol and 0.3 % Triton X-100.

2.8. DNA Extraction (12)

1. Extraction buffer: 200 m*M* Tris-HCl, 50 m*M* ethylene diamine tetraacetic acid (EDTA), 2.2 *M* NaCl, 2% cetyltrimethylammonium bromide (CTAB), 0.06% sodium sulfite, pH 8.0. Autoclave and store at room temperature.
2. Phenol:chloroform:isoamyl alcohol (25:24:1): Store at room temperature in fume hood.
3. 6 *M* NaCl: Autoclave and store at room temperature.
4. 10% Polyvinylpyrrolidone (PVP): Autoclave and store at room temperature.
5. 5% *N*-lauroyl-sarcosine: Autoclave and store at room temperature.
6. 20% CTAB: Autoclave and store at room temperature.
7. TE: 10 m*M* Tris-HCl, 1 m*M* disodium salt (EDTA), pH 8.0.

3. Methods

3.1. Node Explants Culture (see Fig. 1)

All in vitro cultures are incubated at 25°C under a 16-h photoperiod at 36 μmol/m^2/s provide by cool white fluorescent lamps.

1. Shoots (5–8 cm in length) are harvested from mother plant growing in green house.
2. Shoots are surface sterilized for 15 min in sodium hypochlorite (1% available chloride) and one drop of Tween-20 and then rinsed twice for 10 min each in sterilized water.
3. Leaves are removed carefully from the nodes (*see* **Note 2**).
4. The first three nodes below the shoot meristem are cut into 1-mm thick explants, each containing a node and leaf scar (*see* **Fig. 1**).
5. Explants are cultured on Medium I for 10 d.
6. Node explants are cut into eight radial segments and subculture onto Medium II for 4 wk.
7. The regenerated shoots are subcultured onto Hyponex medium.

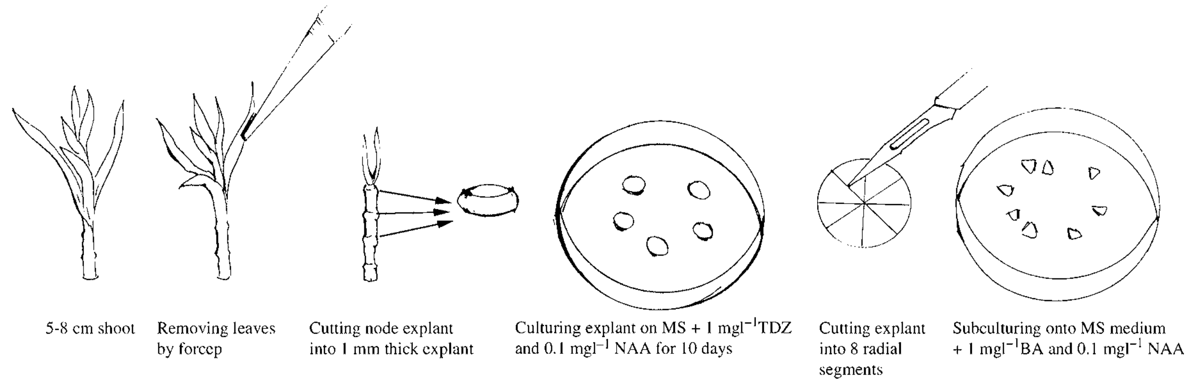

Fig. 1. Node explants preparing and culture step for adventitious shoot induction.

3.2. Explant and Agrobacterium Culture Preparation

1. For genetic transformation, prepare carnation node explants as in **Subheading 3.1., steps 1–4** but preculture them on Medium I for 3 to 5 d.
2. A frozen *Agrobacterium* culture is inoculated into liquid LB medium and incubated overnight at 27°C on a rotary shaker (120 rpm).
3. Solid LB medium is streaked with bacteria suspension and incubated at 27°C for 1 to 2 d. These bacteria can be kept for 1 mo at 4°C.
4. *Agrobacterium* culture from LB solid medium are cultured overnight in 20 mL of LB medium containing 50 mg/L kanamycin and 50 mg/L rifampicin at 27°C on a rotary shaker (120 rpm).
5. One milliliter of *Agrobacterium* is transfered to 1.5-mL Eppendorf tube and centrifuged for 1 min at 2000*g*. The supernatant is removed and the pellet resuspended thoroughly in 10 m*M* glucose and 100 μ*M* AS and centrifuged twice.
6. The density of bacteria is adjusted to 0.5 (OD_{600}) with 10 m*M* glucose and 100 μ*M* AS.

3.3. Agrobacterium Infection and Co-cultivation (see Fig. 2)

1. Node explants are submerged into the *Agrobacterium* suspension for 10 min (*see* **Note 3**).
2. Node explants are blotted briefly on sterilized filter paper.
3. Transfer the infected node explants onto 3 layers of filter paper soaked with water and 50 μ*M* AS in a 90-mm Petri dish.
4. Node explants are co-cultivated with *Agrobacterium* for 5 d in dark conditions at 25°C.

3.4. Selection

1. After co-cultivation, the node explants are cut into eight radial segments and subculture onto the MS selection medium (containing 20 mg/L G418).
2. The explants are subcultured every month onto a fresh MS selection medium with step-wise increased G418 concentrations from 20 mg/L to 30 mg/L and 40 mg/L.
3. Antibiotic resistant shoots are harvested from explants and culture on Hyponex selection medium, until transformed shoots big enough (about 4–6 cm) for acclimation and greenhouse growth (*see* **Notes 4** and **5**).

3.5. Plant Acclimation and Greenhouse Care

1. Carnation shoots are acclimatized by cutting 3- to 4-cm length shoots, placing them into cutting media, and maintaining under shading with high humidity condition for 2 wk.
2. The rooted cuttings are transplanted into 20-cm diameter pot and grown in and special greenhouse for genetic transformation plants (closed glasshouse at 25°C under natural light intensity and photoperiod). Water moderately with water and fertilize with by 14:14:14 control release fertilizer (Nutricote) every 2 mo. The plants flower after 4 to 5 mo.

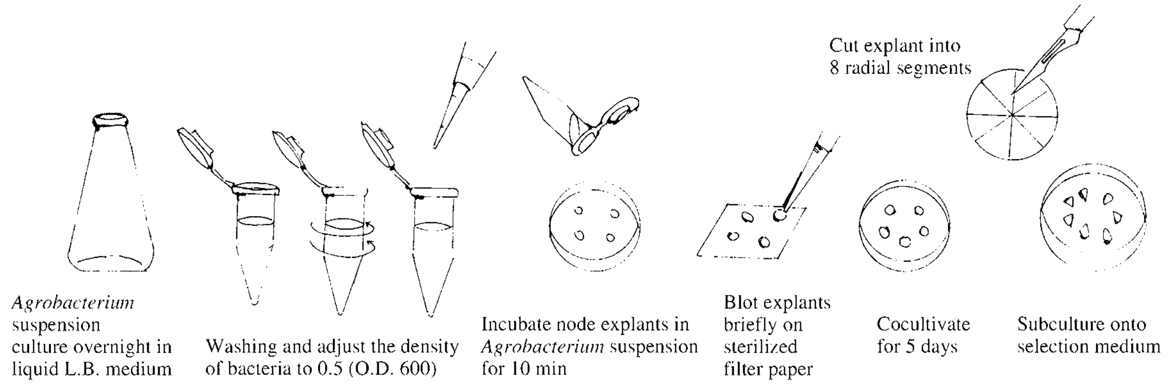

Fig. 2. *Agrobacterium* preparation and transformation procedure of carnation node explants.

3. To prove stable genetic transformation, carnation flowers are covered with bags and pollinate by hand pollination. When seeds are dry, harvest and sow on filter paper, harvest seedling and subject to GUS assay (*see* **Note 7**).
4. The transgenic carnation plants are maintained by tissue culture technique.

3.6. Analysis of Transgenic Plants

All antibiotic resistant plants need to be confirmed for their transgenic nature by GUS assay, polymerase chain reaction (PCR) and Southern blotting analyses.

3.6.1. GUS Histochemical Assay

For investigation of the success of genetic transformation protocol, GUS histochemical assay can be performed at different stages of transformation.

1. *Agrobacterium* infected node explants are sampled at 7thd and 28thd after cocultivation.
2. Incubated the node explants in X-Gluc solution overnight at 37°C.
3. Node explants are de-greened by 70% ethanol.
4. X-Gluc stained node explants are sectioned to 100 µm by using a microslicer (DTK1500, DOSAKA EM Co.Ltd).
5. GUS expressing areas are investigated under light microscope.
6. GUS histochemical assays are performed again when the antibiotic resistant shoots are harvested from node explants and when the plants flower (*see* **Note 6**).

3.6.2. DNA Extraction (Aljanabi Protocol With Modification) (12)

1. Young leaves (10 g) from two greenhouse-grown carnations are picked in the early morning to prevent polysaccharide accumulation in sample.
2. Grinded after quick freezing it in a mortar with liquid nitrogen and place the ground tissue powder into 50-mL centrifuge tubes.
3. Extraction buffer (4 mL/g of leaves weight) is added and using an Ultra-Turax homogenizer with a large dispersing element, for a few seconds.
4. Two milliliters of 5% *N*-lauroyl-sarcosine, 2 mL of 10% PVP and 2 mL of 20% CTAB are added. Mix well by inversion.
5. Samples are incubated for 30 to 60 min at 65°C in a water bath.
6. Samples are mixed by inversion 3 to 4 times during incubation.
7. Samples are taken from the water bath and cooled down to room temperature.
8. An equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol is added and mixed by inversion.
9. Samples are centrifuged the tubes at 3000*g* for 10 min at 4°C.
10. The supernatants are transferred to clean tubes and added an equal volume of isopropanol followed by 2 mL of 6 *M* NaCl.
11. Incubate at 20°C for at least 1 h.
12. Fish out the DNA with a glass pipet and transfer to a fresh tube containing10 mL of 70% ethanol. Alternatively, spin down the DNA pellet briefly, wash with 10 mL of 70% ethanol. If DNA amounts are high enough the fishing out of DNA with a

glass pipet is recommend because the quality of DNA will be better than with the spin down method.

13. DNA is resuspended in 100 µL of TE.

4. Notes

1. This protocol was used for five genotype of carnation. Although *Agrobacterium* could transfer T-DNA into all genotypes, the transformation efficiency still depended on genotype. Susceptible genotypes such as Otome could produce 95 transformed shoots from 100 node explants. In genotypes which are not susceptible to *Agrobacterium*, such as Tanga, only 7 transformed shoots from 100 node explants were produced. One interesting thing is that the number of transformed shoots does not depend on the shoot regeneration ability of carnation. Cultivar Otome produced fewer shoots from node explants than cultivar Tanga, but it could produce more transformed shoots than Tanga.
2. During preparation of node explants, it is important to remove leaves from the shoot by tearing not cutting, because the adventitious shoots will regenerate only from the tearing scar surface, not the cutting surface.
3. Before incubateing with *Agrobacterium* suspension, blot explants briefly on sterile filter paper if the surface of the node explants is too wet.
4. The selection by Hyponex medium containing 80 mg/L G418 is also suitable for transformed shoot selection. Any shoots surviving this selection pressure tested positive by GUS, polymerase chain reaction (PCR), and Southern blot analyses (*10,13*).
5. This protocol can produce transformed shoots (greater than 0.5 cm) within 4 to 6 mo. From this protocol we successfully transferred genes to five genotypes of carnation (Otome, Killer, Master, Tanga, and Laurella). This protocol also works with other genotypes of carnation. In addition, this protocol can be used for genetic transformation of in vitro leaf explants of carnation; however, transformation is low when compareed with node explants. This protocol was still effective when used to transfer genes into regenerable calli of *Dianthus hybrida* (Telstar scarlet).
6. Whenever performing the GUS assays, nontransformed explants should be included as negative controls to rule out the background effect during the staining process.
7. Generally carnation is propagated by vegetative propagation; a good transformed mother plant can be used for propagation by cutting.

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29

Chrysanthemum (*Dendranthema* × *grandiflora*)

Jaime A. Teixeira da Silva

Summary

This chapter provides reproducible methods for the transformation of *Dendranthema* × *grandiflora* 'Shuhou-no-chikara' (standard) and 'Lineker' (spray) stem internode thin cell layers (TCLs) and conventional stem internode explants using *Agrobacterium* strains carrying a binary vector with β-glucoronidase (GUS) reporter and *npt*II selector genes. Transformation efficiencies are reported at the kanamycin selection (callus formation and plantlet rooting), GUS assay, polymerase chain reaction (PCR), and Southern analysis levels. Notes on regeneration improvement through the use of TCLs, as well as the effective use of sonication for both regeneration and generation of transformants, stimulation of *Agro*-infection and elimination of *Agrobacterium*, are included.

Key Words: Acetosyringone (AS); AGL0; CaMV-35S promoter; *Dendranthema*; *npt*II; plant growth regulator (PGR); sonication-assisted *Agrobacterium* transformation (SAAT).

1. Introduction

Chrysanthemum (*Dendranthema* × *grandiflora*) is one of the most important global floricultural assets whose cultivation and improvement/modification by traditional breeding dates back over 2000 yr. The latter part of the 21st century was marked by in vitro regeneration studies, while the past 15 yr have emphasized improvement by genetic transformation ***(1)***. Because of generally low-transformation efficiencies being reported, and because of inconsistencies and repeatability of protocols, regeneration studies have once again come to the forefront. The intricate interlink between the success of *Agrobacterium*-mediated transformation of chrysanthemum and regeneration will be highlighted in this chapter. In almost 50 reported cases of *Agrobacterium*-mediated transformation of chrysanthemum, the initial explant used was: leaf (50%), stem (22%), leaf/stem (14%), roots (2%), and flower parts (2%). The choice of selective agent

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was: kanamycin sulfate (80%), G418 (4%), geneticin (4%), hygromycin (10%), Basta (2%), paramomycin (2%), or none (6%); selection timing was early (76%), late (12%), or unspecified (12%). Furthermore, where kanamycin was the choice of agent, more than half of the studies employed a low (≤25 mg/L) level of initial selection with only 20% of studies increasing the selection level during regeneration, with most (78%) research groups decreasing the selection pressure *(1)*. The choice of *Agrobacterium* strain was: LBA4404 (56%), EHA101/105 (22%), AGL0/1 (20%), A281 (10%), or others (28%); only a single study of *A. rhizogenes* using LBA9402 was reported *(2)*. In these studies, during regeneration the bacterocidal agent used for elimination of *Agrobacterium* was: cefotaxime (64%), vancomycin (26%), carbenicillin (16%), ticarcillin (16%), individually, or in combination. These agents were applied, in most cases (84%), at approx 250 mg/L initially, with 36% of studies decreasing/halving this concentration at later stages of selection. Only a single study reported the use of Sonication-Assisted *Agrobacterium* Transformation, or SAAT *(3)*.

Indeed, the difficulties facing the genetic transformation of chrysanthemum resulting from the choice of explant, selective agent and/or *Agrobacterium*-eliminating bactericide, and the levels and timing of *Agrobacterium* application, as well as the choice of *Agrobacterium* strain are confounded by the reports of the need for wounding *(4)*, chrysanthemum cultivar and *Agrobacterium* strain dependence (reviewed in *[1]*), late transgene (GUS) expression *(5)*, transgene silencing *(6–8)*, chimerism caused by transgene inactivation, primarily by methylation *(9)*, and possible transgene truncations leading to smaller proteins *(10)*.

The protocol within this chapter aims to address these difficulties and shortcomings. Although the protocols described are based on in vitro plantlet stem internode thin cell layers (TCLs), very similar transformation frequencies at the GUS, polymerase chain reaction (PCR), Southern and Western levels can be obtained for leaf material; but the ease of explant manipulation and repetitiveness of the protocol is much higher in the case of the former. Moreover, these small explants allow for the controlled regeneration of transgenic organs (shoot, root, somatic embryo, friable callus), depending on the chosen medium *(11)*.

In our system, stem internode TCLs or conventional longitudinally-bisected stem explants are co-cultured for 2 d with 100 m*M* acetosyringone (AS)-supplemented overnight bacterial culture on optimized regeneration medium. In the case of SAAT, explants are placed in the same co-cultivation medium in 1.5-mL Eppendorf tubes and sonicated for 2 to 5 min. Following co-cultivation, *Agrobacterium* infected explants are placed on kanamycin and cefotaxime-supplemented medium, and emergent shoots are rooted in vitro and 100% acclimatized to the greenhouse.

Transformation efficiencies, measured at different levels are: 31% explants forming callus on 30 mg/L kanamycin on SIM_{IV}; 21% GUS positive (and PCR positive) in vitro plants (old, middle-aged, and young leaves staining positive for GUS) growing on 30 mg/L kanamycin on SIM_{IV}; 8% GUS positive (and PCR positive) plants (old, middle-aged, and young leaves staining positive for GUS) 2 mo after growing in the greenhouse; 8% Southern positive in vitro plants (old, middle-aged, and young leaves staining positive for GUS) growing on 30 mg/L kanamycin on SIM_{IV}; 1% Southern positive plants (old, middle-aged, and young leaves staining positive for GUS) 2 mo after growing in the greenhouse. An overall transformation efficiency of approx 1% (10 Southern positive from 1000 initial TCLs) can be obtained using this protocol while an almost 2% transformation efficiency is possible using SAAT.

2. Materials

All reagents are tissue culture or molecular biology grade. All solutions are prepared in sterile, autoclaved, double-distilled water (SDDW), 18.2 MΩ-cm, organic content <5 ppb. Autoclaving is always for 15 min at 121°C and 103 kPa.

2.1. Plant Materials

Protocols were developed with two leading Japanese cultivars, 'Shuhou-no-chikara'—a standard—and 'Lineker'—a spray-type chrysanthemum [Dendranthema × grandiflora (Ramat.) Kitamura]. Both greenhouse and in vitro plant material serve equally well as initial explant sources (culture conditions for both are provided below), but in vitro material is recommended to avoid viral contamination.

2.2. Plant Culture

1. Plant growth regulator (PGR) stocks: 6-benzyladenine (BA) and α-naphthalene acetic acid (NAA) at 100 mg/L each. Dissolve 100 mg BA or NAA in 1 *N* NaOH and bring to volume in SDDW. Do not adjust the pH. Keep both BA and NAA stocks at 4°C for up to 6 mo.
2. Optimized in vitro shoot induction medium (SIM_{IV}): Add 2 mg/L BA, 0.5 mg/L NAA, and 40 g/L sucrose to full-strength Murashige and Skoog ([MS] macro- and microelements, and vitamins *[12]*) medium. Adjust pH to 5.7 and autoclave. Commercial MS powder (Sigma-Aldrich) provides the same results.
3. In vitro shoot regeneration medium (SRM_{IV}) and greenhouse shoot regeneration medium (SRM_{GH}): 3 g/L Hyponex® (Japan) + 20 g/L sucros. Adjust pH to 5.7 and autoclave.
4. Optimized greenhouse shoot induction medium (SIM_{GH}): Prepare as SIM_{IV} but add 1 mg/L BA, 1 mg/L NAA, and 20 g/L sucrose. Adjust pH to 5.7 and autoclave.
5. All plant media are adjusted to between pH 5.7 and 5.8 using 0.1 *M* NaOH or 1 *N* HCl before adding agar (Wako) at 8 g/L and autoclave.

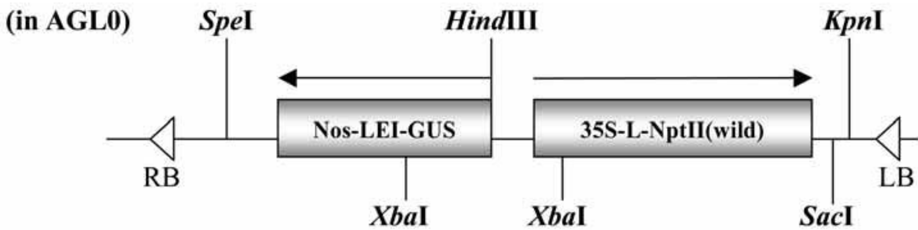

Fig. 1. Diagram of pKT3-plasmid T-DNA regions. *RB*, right border; *LB*, left border; P35S, 35S promoter; Pnos, nopaline synthase promoter; *GUS*, β-glucoronidase gene; *npt*II, neomycin phosphotransferase gene; L and E, enhancers; int, intron. *Hin*dIII, *Kpn*I and *Spe*I, restriction enzyme sites.

2.3. Agrobacterium *Strain, Culture, and Stocks*

1. *A. tumefaciens* strain AGL0 harbors a binary plasmid pKT3 *(1)* (Kirin Breweries Biotechnology, Japan) (*see* **Fig. 1**).
2. Luria-Bertani (LB) medium: 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl. For plating, add 15 g/L bactoagar, and adjust pH to 5.3. Autoclave and store at 4°C.
3. Antibiotics stocks: Kanamycin and rifampicin at 10 mg/mL each, cefotaxime (Claforan®) at 100 mg/mL. Filter-sterilize and store at –20°C for up to 6 mo.
4. AS (3,5-dimethoxy-4-hydroxy-acetophenone; Sigma-Aldrich) stock (100 m*M*): Dissolve AS in dimethyl sulfoxide (DMSO) or in 100% EtOH. Store at 4°C in foil for up to 6 mo. AS crystallizes at 4°C but is liquid at room temperature. Add 1 μL AS stock to 1 mL *Agrobacterium* medium or 1 mL to 1 L of co-cultivation medium for 100 m*M* final concentration.

2.4. Other Supplies

1. Potting soil: 7:3 masa:compost mix. Masa (Japan) is decomposed granite.
2. Hyponex® medium (powder): 6.5:6:19 (N:P:K).
3. JiffyMix® MM 350: pH 5.0 to 6.0, Jiffy-Garden Series (Scotts Co.).
4. Rooton® (Japan): Commercial rooting hormone (IBA) powder.
5. Kanuma soil: River sand.

3. Methods

3.1. In Vitro Explant Preparation From Ex Vitro Material

In vitro shoot cultures are initially established from greenhouse stem explants. Often chrysanthemum breeders who wish to conduct genetic transformation of a cultivar or variety that they have observed in a greenhouse need to first acclimatize it to in vitro conditions. This regeneration protocol is also essential to the success of conversion of transgenic plants from ex vitro to in vitro *(13–17)*.

1. Excise terminal 10 to 15 cm of greenhouse mother plants with a sterile lamina to avoid possible transfection of viruses/viroids from plant to plant. Actively-growing mother plants, preferably 30 to 40 cm high (approx 2 mo old) should be used. Place cut-ends into SDDW.
2. Remove all leaves, and wash naked stems in a commercial dishwashing liquid solution for 1 min.
3. Rinse thoroughly under luke-warm SDDW, and rinse 3 times with SDDW.
4. Transfer stems to a 1% NaOCl (1% active Cl) or $Ca(ClO_2)$. Add 1% (v/v) polyoxyethylene sorbitan monolaurate (surfactant), or Tween-20. Stir for 15 min under laminar flow conditions and rinse 3 times with SDDW.
5. Remove apical 2 cm from sterilized stems, as well as any old basal tissue (typically exhibiting white, lignified tissue) using a sterilized 0.22 stainless-steel surgical blade (Feather, Japan).
6. Cut remaining internode stem tissue transversally into 3- to 5 mm thick slices, cut in half again (do this to obtain semi-cylindrical explants, 3–5 mm in length).
7. Place sections adaxial surface down onto filter paper overlaid in a Petri dish (90 × 15 mm) containing SIM_{GH} medium.
8. All in vitro cultures are maintained at 25°C, 16-h photoperiod, light intensity 45 µmol/m^2/s (PGF lamps). Shoot primordia will form within 6 to 8 d, and harvestable shoots will emerge within 3 to 4 wk of initial culturing. There is no need for sub-culturing.

3.2. Preparation of Explant Material for Transformation

1. Excise the shoots (at least two fully developed leaves) that were formed from the explants (*see* **Subheading 3.1.**) into Magenta-7 plant boxes (4 plants/box) containing SRM_{GH} under the same growth conditions (*see* **Subheading 3.1.**). Rooting will occur within 1 wk, and approx 8-cm plants will develop within about 60 d.
2. Two days before the transformation epxperiment, prepare TCLs from in vitro plantlets as follows. Cut TCLs (approx 200–500 µm thick, 1–1.5 mm in diameter) from the second or third stem, from the apex, internode using sterilized Feather-S blades (0.1-mm thick; Feather, Japan). Place TCLs onto SIM_{IV}, 20 TCLs/petri dish, which is then sealed with Parafilm. Incubate under light at 25°C as described above.
3. In order to induce roots from in vitro induced shoots, shoots derived from SIM_{IV}, having three nodes, are cut and placed onto SRM_{IV} under the same growth conditions as described in **Subheading 3.1.** (*see* **Note 1**).

3.3. Agrobacterium *Culture Preparation*

1. One day before the transformation experiment, inoculate a single colony of *A. tumefaciens* AGL0 carrying pKT3 in 20 mL LB medium + 50 mg/L kanamycin. Shake at 120 rpm for 16 to 20 h at 27°C.
2. Centrifuge 1 mL of overnight culture (11,500*g* or 8000 rpm for 3 mins). Resuspend the *Agrobacterium* pellet in 1 mL of 10 m*M* glucose + 100 m*M* AS. Adjust OD_{540} to between 0.4 and 0.5.

3.4. *Agrobacterium Infection and SAAT*

1. Move precultured TCLs on a filter paper (10 explants/petri dish) overlying non-selective MSIV for 2 d, and apply 10 µL of *Agrobacterium* culture to each TCL by pipetman. Place under the same growth conditions as in **Subheading 3.1.**
2. Co-culture on nonselective MSIV for 3 d (*see* **Note 2**). Place under the same growth conditions as in **Subheading 3.1.**
3. In the case of SAAT, pre-culture TCLs or longitudinally-bisected stem internode explants on MSIV for 24 to 36 h.
4. Place 10 TCLs or stem explants in 1.5-mL Eppendorf tubes containing 1 mL of *Agrobacterium* culture suspended in 10 m*M* glucose (or MSIV) and 100 m*M* AS. Place tubes at 27°C in a water bath sonicator (Iuchi®, Japan) at 60 Hz, for 2 or 5 min for TCLs or stem explants, respectively.
5. Following sonication, blot-dry the TCLs on sterilized filter paper. Place on non-selective MSIV for a 2-d co-cultivation period with *A. tumefaciens.*

3.5. *Selection of Putative Transformants and Regeneration*

1. Following the co-cultivation (with or without SAAT treatment), transfer the infected TCLs to SRMIV + 30 mg/L kanamycin + 250 mg/L cefotaxime (Claforan®) for 1 wk. Then transfer onto SRMIV + 30 mg/L kanamycin + 125 mg/L cefotaxime. Subculture every 2 wk (*see* **Note 3**).
2. In the case of the control (3–4 wk later), or in the case of *Agrobacterium* infected explants (4–6 wk later) shoots can be seen to emerge from the epidermis or the cut surface of the explant cultured on SRMIV. Excise the resulting shoots with at least 3 fully developed leaves and place them into a Magenta box containing SRMIV (plus 30 mg/L kanamycin and 125 mg/L cefotaxime) under the same growth conditions (as described in **Subheading 3.1.**). Root primordia will form within 6 to 7 d, and roots will emerge and develop within 2 wk.

3.5. *Acclimatization*

The most important verification step in the success of the transformation process is the successful acclimatization and flowering (main target) of in vitro plants. All in vitro plantlets, nontransgenic or transgenic, are placed in a greenhouse, or a phytotron, respectively (*see* **Note 4**).

1. Wash rooted in vitro plantlets (approx 2 nodes, 4 leaves) under tap water. Place the plantlet in a 30-cm diameter pot containing JiffyMix® soil (25°C, natural photoperiod and light intensity).
2. For plantlets without a root system, dip terminal 3 to 4 cm cutting with 3 to 4 nodes and denuded of leaves, into Rooton® (Japan) and place in Kanuma soil at 25°C. This method is also ideal for the vegetative propagation of greenhouse material.
3. Acclimatize plantlets indoors for 2 wk by covering with an aerated plastic bag, watering daily. Success rate is usually between 98 and 100%.

4. When plantlets are 6 to 8 cm tall, transfer to 11-cm diameter plastic pots with autoclaved potting soil. Pot 4 plants/pot (2 L, 25-cm diameter, clay). Transplant rooted cuttings 1- to 2 wk later and water with Hyponex® medium once/wk, all-yr round.
5. For both nontransgenic and transgenic plants, place at 15 to 25°C under long-day conditions (4-h night-break from 10 PM to 2 AM) in winter, or natural long days in summer to maintain the vegetative state.
6. Induce flowering of any plant under short-day conditions (10 h/d using black out screens or tunnel cover) (*see* **Note 5**). Water thoroughly once every 4 to 5 d in winter, or 1 to 2 d in summer. Add Hyponex® fortnightly to both vegetative and flowering plants.
7. Apply appropriate pesticides should aphids or red spider (most common) appear.

3.6. Morphological Scoring and Transformation Efficiencies

1. The percentage and position of GUS expression is recorded as the number of GUS focal points (GFPs) and blue-staining areas (BSAs).
2. For any one treatment, and for any one plasmid construct, 50 Petri dishes with 10 TCLs/stem explants are used, (i.e., 500 initial explants). Transformation efficiency is calculated as $[(Y/Z) \times 100]\%$, where Y = the number of actively dividing calli or rooted in vitro cuttings on 30 mg/L selective medium (SIMIV or SRMIV, respectively), the number of GUS-spot or GUS-stain-containing explants (transient expression), or GUS expressing rooted plants (stable expression) on selective SRMIV (in young, middle-aged, and old leaves), and the number of plants with correct banding in PCR or Southern analysis; Z = the total number of initial explants (500) (*see* **Note 6**).

4. Notes

1. TCLs have been shown to be useful in controlling desired organogenesis, more than conventional explants where multiple organogenic programmes (shoot, root, callus, somatic embryo) may arise ***(13–15)***. These programs may be induced by numerous media with single or multiple PGR applications. Only the ideal medium is presented here. TCLs also eliminate genotype-dependence, as seen with several spray and standard-type cultivars.
2. Because of the very thin size of TCLs, rapid overgrowth by *Agrobacterium* may occur. In order to minimize this effect, change the position of the explants daily, swabbing off excess bacterial growth onto sterile filter paper, or remove the explant completely. The incidence of bacterial overgrowth may reach up to 6 to 8% of explants, but generally SAAT treated-TCLs or conventional stem explants suffer lower percentages of infection.
3. In the case of AGL0, co-cultivation should not exceed 3 d. *A. tumefaciens* shows different patterns of growth on TCLs cultured with supplementary AS on filter paper and in the presence or absence of PGRs; the presence of PGRs strongly enhances *Agro*infection while the presence or absence of filter paper affects both the effectiveness of regeneration and *Agro*infection ***(16)***. Pre- and co-culture of TCLs should be on non-selective MS_{IV}.

4. Transgenic plants are placed in isolation in a phytotron because there may be the risk of pollen transfer to wild or other cultivated plants. To test for morphological stability, the following parameters should be measured in both control and transgenic greenhouse plants: number of leaves, number of flower buds (for spray-type), plant height, number of disk and ray florets, and flower color.
5. No additional lighting is required in the summer but provide long days in the winter by using high pressure sodium lamps (Philips HPS 400W) to an average PAR of between 50 and 200 μmol/m^2/s (depending on transmission percentage), although ideally the daily PAR light integral (measure with a Kipp solarimeter) should be 15.42 MJ/m^2. To aid spray development interrupt short d by exposing the plants to 10 long d, then place them back under short day conditions for normal flower development.
6. A decrease in the explant induction or shoot regeneration capacity of explants may reflect the inhibitory nature of *Agrobacterium* on shoot induction. T-DNA inserts from the left border to the right border into the host genome by preferential attachment to A-T-rich regions and by forming a short DNA duplex ***(18)***. Consequently, higher *npt*II transformation efficiencies (relative to other transgenes within the same cassette) at most levels (PCR, Southern), reflect a partial or incomplete insertion of the transgene cassette into the plant genome.

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Orchids (*Cymbidium* spp., *Oncidium,* and *Phalaenopsis*)

Ming-Tsair Chan, Yuan-Li Chan, and Sanjaya

Summary

Recent advances in genetic engineering have made the transformation and regeneration of plants into a powerful tool for orchid improvement. This chapter presents a simple and reproducible *Agrobacterium tumefaciens*-mediated transformation protocol and molecular screening technique of transgenics for two orchid species, *Oncidium* and *Phalaenopsis*. The target tissues for gene transfer were protocorm-like bodies (PLBs) derived from protocorms, into which constructed foreign genes were successfully introduced. To establish stable transformants, two stages of selection were applied on the PLBs co-cultivated with *A. tumefaciens*. About 10% transformation efficiency was achieved in *Oncidium* orchid, as 108 antibiotic resistant independent PLBs were proliferated from 1000 infected PLBs. In *Phalaenopsis* orchid about 11 to 12% of transformation efficiency was achieved by using the present protocol. Different molecular methods and GUS-staining used to screen putative transgenic plants to confirm the integration of foreign DNA into the orchid genome were also described in detail. The methods described would also be useful for transformation of desired genes into other orchid species.

Key Words: *Agrobacterium*; *Oncidium*; *Phalaenopsis*; protocorm-like bodies (PLBs); plant regeneration; microparticle bombardment.

1. Introduction

Orchids are a group of interesting plants and represent an aristocracy in floriculture. *Oncidium* and *Phalaenopsis* are indigenous and popular commercial orchid species of the Taiwan floriculture industry. Taiwan is also one of the most important breeding and export country of orchids, especially for the *Phalaenopsis* orchid. Sales in 2003 accounted for approx \$20,250,000 (according to statistics from the Bureau of Foreign Trade in Taiwan). In the past, conventional breeding strategies have resulted in the production of orchid hybrids with valuable traits, including new flower pigments and novel flower shapes. However, the long life cycle and lack of variants in naturally occurring lines have limited the improvement of orchids. In

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contrast, the gene-transfer techniques provide a new avenue for the production of new orchid varieties with important characteristics, such as flower pigmentation, disease and pest resistance. In this direction tissue culture manipulation, plant regeneration and breeding techniques of different orchid plants have been well established (*1–4*), and provide valuable setups for gene transformation.

The last two decades have seen significant development in the plant transformation technologies for the insertion of foreign DNA into plant genome. The most popular transformation systems are *Agrobacterium*-mediated transformation and microparticle bombardment (biolistics). By utilizing these transformation systems, transgenics have been successfully established in a variety of plant species such as maize (*5*), rice (*6*), wheat (*7*) and barley (*8*). To date, few studies have been demonstrated the gene transfer techniques for orchids (*9–12*). However, most of the reported protocols describe the transfer of reporter genes via microparticle bombardment. The transgene silencing, instability, and rearrangements are more evident with the biolistic method than the *Agrobacterium* method of gene transformation (*3*). The reliable gene delivery method for *Oncidium* and *Phalaenopsis* orchids via *Agrobacterium*-mediated transformation and some key points were discussed.

2. Materials

2.1. Propagation of PLBs

1. T2 medium (1 L): 3.5 g Hyponex No. 1 (N:P:K fertilizer, Hyponenex Co., Taiwan), 1 g tryptone, 0.1 g citric acid, 20 g sucrose, 1 g charcoal, 20 g sweet potato, and 25 g banana (peal outer coat and prepare fine paste by using kitchen mixer). The pH of the medium is adjusted to 5.5 with 0.1 *N* HCl or 0.1 *N* NaOH before autoclaving and gelling with 0.3% Phytagel® (Sigma). Molten medium (50 mL) is dispensed into 400-mL culture bottles. The culture bottles are capped with lids and autoclaved at 15 lb/inch^2 pressure at 121°C for 20 min.
2. G10 medium (1 L): 4.3 g Murashige and Skoog (MS) salts, 1 g tryptone, 20 g sucrose, 1 g charcoal, 65 g potato tubers, and 3 g Phytagel. Adjust to pH 5.4 and autoclave (*see* **Note 1**).

2.2. Agrobacterium *Culture Media*

1. YEP medium (1 L): 10 g peptone, 10 g yeast extract, and 5 g NaCl, pH 7.0.
2. AB medium (1 L): 7.25 g KH_2PO_4, 10.25 g K_2HPO_4, 0.15 g NaCl, 0.15 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2$, 5 g glucose, 2.5 mg $FeSO_4$, and 8 g agar. Supplement with the optimal concentration of antibiotics.

2.3. Nutrient Media and Antibiotics

2.3.1. For Oncidium

1. Pre-infection medium: G10 medium plates without charcoal, but with 200 μ*M* acetosyringone (AS) and a nurse culture of tobacco suspension cells.

2. Co-culture medium: T2 solid medium with 5% glucose and 200 μ*M* of AS.
3. Washing medium: MS and G10 liquid medium supplemented with 200 mg/L timentin.
4. Selection medium (1 L): 4.5 g Knudson C orchid medium salts (Duchefa BioChemie, Haarlem, The Netherlands, cat. no. K 0215), 20 g sucrose, 1 mL MS vitamins (1 mg thiamine hydrochloride, 1 mg pyridoxine hydrochloride, 10 mg nicotinic acid, 100 mg *myo*-inositol), and 3 g Phytagel. Supplement with 200 mg/L timentin and the optimal concentration of antibiotics (pH 5.4).

2.3.2. For Phalaenopsis

1. Pre-infection medium: T2 solid medium supplemented with 200 μ*M* AS (Sigma).
2. Co-culture medium (1 L): 4.31 g MS salts (Sigma), MS vitamins, 20 g sucrose, and 0.3% Phytagel. Adjust to pH 5.4 before autoclaving and add 5% glucose and 200 μ*M* of AS (filter-sterilize and mix with medium before pouring into culture bottles.
3. Washing medium: MS medium supplemented with 200 mg/L timentin.
4. Selection medium: T2 medium supplemented with 200 mg/L timentin and the optimal concentration of antibiotics.
5. 0.5 *M* sucrose.

2.4. Genomic DNA and Total RNA Isolation

1. DNA extraction buffer: 3% (w/v) Hexadecyltrimethyl-ammonium bromide (CTAB) (Sigma), 1.42 *M* NaCl, 20 m*M* ethylene-diaminetetraacetic acid (EDTA) (Sigma), 100 m*M* Tris-HCl, pH 8.0; 2% (w/v) polyvinylpyrrolidone (PVP-40) (Sigma Chemical, St. Louis, MO), and 5 m*M* ascorbic acid. Store at room temperature.
2. 5% Cetyl trimethyl ammonium bromide (CTAB) solution: 5% CTAB, 0.7 *M* NaCl.
3. 7.5 *M* NH_4OAc.
4. Absolute/isopropyl alcohol, 70% ethanol, and 75% ethanol. Store at −20°C.
5. Chloroform and liquid nitrogen.

3. Methods

3.1. Propagation of Protocorm-Like Bodies (PLBs)

1. The PLBs of *Oncidium* and *Phalaenopsis* orchids are chopped and cultured independently on G10 and T2 medium (arrange 90–100 PLBs per Petri dish).
2. PLBs are incubated for a 16-h photoperiod at 25°C with light provided by cool white fluorescent lamps with an intensity of 200 μE/m^2/s.

3.2. Estimation of Antibiotic Concentration for Selection

1. Culture PLBs of *Oncidium*/*Phalaenopsis* orchid on G10/T2 medium supplemented with various concentrations of antibiotic (*see* **Note 2**).
2. Incubate PLBs under 16-h photoperiod at 25°C for 4 to 6 wk, and record the survival rate of PLBs for every week.
3. The medium with lowest concentration of antibiotic, on with no PLBs are survived, will be the optimum concentration for selection.

3.3. Preparation of Agrobacterium Culture

1. Take out *Agrobacterium tumefaciens* containing a binary vector from −80°C stock, streak on YEP solid medium supplemented with optimal concentration of antibiotic and incubated at 28°C for 48 to 72 h.
2. Pickup a single colony of *A. tumefaciens* and culture in 5 mL AB liquid medium supplemented with the optimal concentration of antibiotic and incubate overnight at 28°C.
3. Inoculate 200 to 300 µL overnight-grown bacterial culture to 50 mL AB medium supplemented with the optimal concentration of antibiotic and incubate overnight at 28°C (use 250-mL flask).
4. Terminate *A. tumefaciens* culture growth when it reach to A_{600} = 0.8–1.0, centrifuge at 3000*g* for 10 min and resuspend the pellet in 20 mL MS medium supplemented with 5% glucose and 200 µ*M* of AS (adjust the final OD of the culture after resuspend to A_{600} = 0.5–0.6).

3.4. Transformation of Oncidium PLBs

Different transformation stages of *Oncidium* orchid are shown in **Fig. 1**.

1. Select newly differentiated PLBs (45-d old culture) and pre-treat with 0.5 *M* sucrose for 2 h in sterilize Petri dish (all explants should submerged) (*see* **Fig. 1A** and **Note 3**).
2. Transfer pre-treated PLBs on G10 medium and incubated at 26°C in dark for 7 d.
3. Pool all PLBs together in a Pertri dish; add *Agrobacterium* culture and allow to sit for 30 min in the dark on shaker (optional). Drain out *Agrobacterium* culture, surface dry PLBs on sterilized filter paper (2–3 min), and transfer healthy PLBs onto co-culture medium (*see* **Subheading 2.3.1.**).
4. Spread 1 mL tightly packed 3-d-old freshly subcultured tobacco suspension cells on G10 medium with 200 µ*M* AS. Place sterilized filter paper over suspension cells and co-cultivate infected PLBs for 3 d in dark at 26°C (*see* **Fig. 1B**).
5. Wash infected PLBs three times in MS liquid medium supplemented with 200 mg/L timentin at the interval of 30 min on shaker (use 100 rpm) (*see* **Note 4**).
6. Blot dry and transfer PLBs on G10 medium supplemented with 200 mg/L timentin and incubate at 26°C for a 16-h light/8-h dark photoperiod at a light intensity of 200 $\mu E/m^2/s$ for 4 to 6 wk for the development of new PLBs.
7. Subculture healthy and green PLBs on PR medium with the optimal concentration of antibiotics for further selection of putative transformants after two months (*see* **Fig. 1C**).
8. Subculture newly differentiated green PLBs on fresh G10 medium without antibiotics and maintain cultures up to 3 mo (*see* **Fig. 1D,E**) (subculture at the interval of every 4 wk) (*see* **Note 5**).
9. Roots appear after 1 to 2 mo. After 6 to 8 wk plants (3–4 cm in height) with 3- to 6 roots are eventually selected for hardening (*see* **Fig. 1F**).
10. Wash plants thoroughly in running tap water to remove adhered nutrient medium. Transfer to 200 to 300 cm^3 pots (one plant/pot) containing sphagnum moss and acclimatize under greenhouse conditions (25°C temperature and 70–80% relative humidity [RH]).

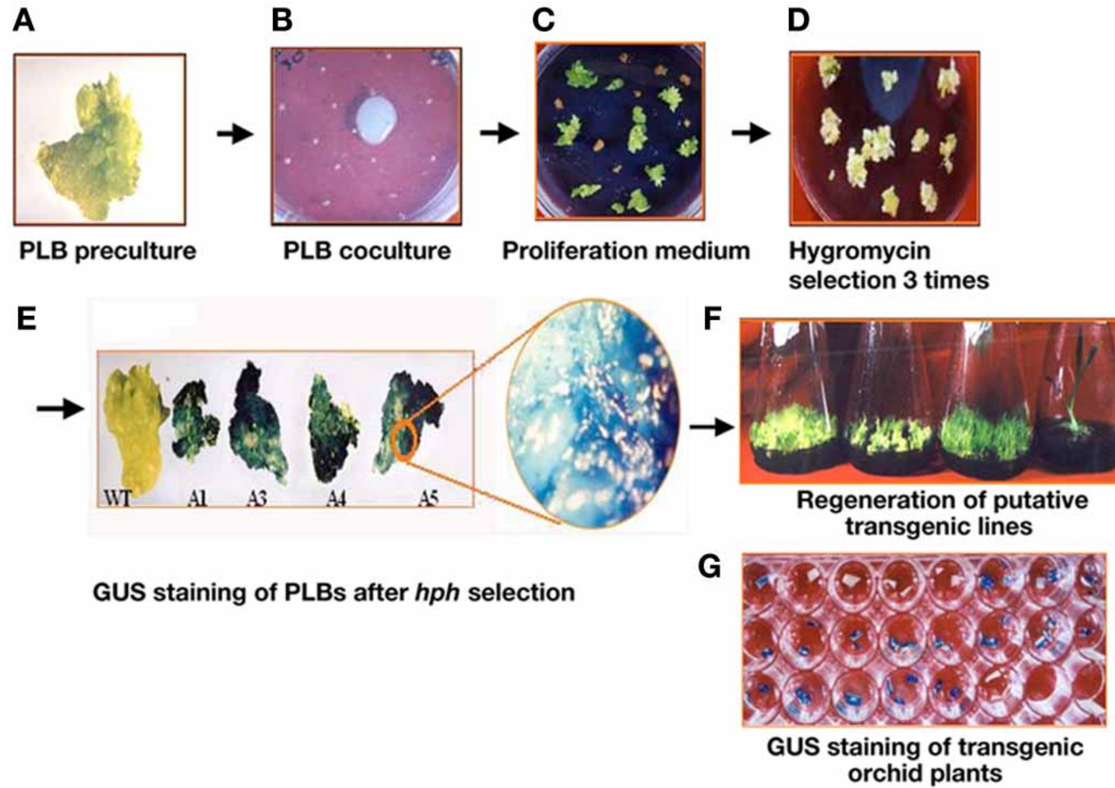

Fig. 1. Different developmental stages of transgenic *Oncidium* orchid through *Agrobacterium* mediated transformation. (**A**) 45-d-old pre-treated PLBs on G10 medium. (**B**) Co-cultivation of PLBs infected with *Agrobacterium* on G10 medium supplemented with 200 µ*M* AS and a nurse culture of tobacco suspension cells for 3 d in dark at 26°C. (**C**) Proliferation of new PLBs on PR medium. (**D**) Newly differentiated green PLBs during third selection on G10 medium with antibiotics. (**E**) GUS staining for putatively transformed PLBs. (**F**) Regeneration of putatively transformed lines. (**G**) GUS staining of transgenic orchid plants.

11. Irrigate plants for every other day, and spray 1 to 2% Hyponex® fertilizer solution (Hyponenex Co., Taiwan) at 15-d intervals to boost the growth of the plants. Interesting lines are maintained and used for breeding purposes, and further multiplication can be performed by using side shoots (tillers). The application of insecticides or pesticides is optional.

3.5. *Transformation of* Phalaenopsis *PLBs*

1. Newly regenerated PLBs (preferably 30-d-old cultures) are chopped and cultured on T2 medium and incubate in the dark for 7 d.
2. Transfer PLBs to fresh T2 medium supplemented with 200 μ*M* AS and continue to leave in the dark for 1 h.
3. Carefully transfer PLBs into *Agrobacterium* culture, and extend infection for 1 h in dark.
4. Co-cultivate blot dried PLBs on T2 solid medium supplemented with 200 μ*M* AS and 5% glucose at 26°C in dark for 3 d.
5. Wash *Agrobacterium* infected explants three times with MS liquid medium supplemented with 200 mg/L timetin at the interval of 30 min on shaker (use 100 rpm) (*see* **Note 4**).
6. Transfer blot dried PLBs to T2 medium supplemented with 200 mg/L timetin and incubate at 26°C with 16-h light/8-h dark photoperiod for 4 wk at a light intensity of 100 to 200 μE/m^2/s.
7. Separate newly differentiated PLBs from the original explant, subculture on T2 medium supplemented with 200 mg/L timentin and the optimal concentration of antibiotics for selection of putative transformants.
8. Transfer actively growing PLBs on T2 medium supplemented with the optimal concentration of antibiotics for the second selection to establish transgenic lines (*see* **Note 6**).
9. After 6 to 8 wk, transfer surviving and actively growing putatively transformed PLBs on fresh T2 medium for shoot elongation.
10. Transfer well established plants (3–4 cm in height and 2–3 leaves) to 200 to 300 cm^3 pots (1 plant/pot) with sphagnum moss and acclimatize under greenhouse conditions (25°C temperature and 70–80% RH).

3.6. *Isolation of Genomic DNA and Total RNA From Putative Transformants*

1. PLBs are ground to a fine powder in liquid nitrogen, and samples were further homogenized for 2 min with 300 μL of DNA extraction buffer.
2. Samples were heated at 65°C for 15 min and then extracted with chloroform.
3. For DNA isolation, $^1/_5$ volume of 5% CTAB solution was added to the aqueous phase.
4. The samples were mixed well and heated at 65°C for 15 min and then extracted with chloroform.
5. Two volumes of ethanol were added to the supernatant, incubated at −20°C for 15 min and centrifuged for 15 min.
6. Wash pellet in 70% ethanol, dry and dissolve pellet in TER and quantitate the amount of DNA by using spectrophotometer.

7. For RNA isolation, add $^1/_2$ volume 7.5 *M* NH_4OAc to the aqueous phase obtained from **step 2.** Incubate on ice for 15 min and centrifuged at 16,000*g* for 10 min.
8. Wash the pellet in 75% ethanol and centrifuge at 7500 rpm for 5 min at 4°C.
9. Briefly dry the RNA pellet, dissolve in RNAase-free water and store at −80°C (*see* **Note 7**).

4. Notes

1. Charcoal is not a necessary ingredient for PLBs propagation; however, incorporation may help in regeneration.
2. The antibiotics used for transformation depends on the selection marker of the binary vector; the concentration of antibiotics may vary from the test orchid cultivars.
3. PLBs of *Oncidium* with 0.2-mm in size (without any shoot) are the most ideal plant material for transformation.
4. Overgrowth of *Agrobacterium* needs to be prevented to avoid the death/desiccation of infected PLBs. While washing, use the same concentration of antibiotic in MS liquid medium.
5. GUS staining of putatively transformed PLBs is optional at this stage.
6. Cull out brown/dried original PLBs from the medium, otherwise it may cause *Agrobacterium* growth.
7. For isolation of total protein, GUS staining, Southern, Northern, and Western blot analysis, follow the standard procedures.

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Petunia (*Petunia hybrida*)

W. Kevin Lutke

Summary

Petunia hybrida genetic transformation continues to be a valuable tool for genetic research into biochemical pathways and gene expression, as well as generating commercial products with varying floral colors. In this chapter, we describe a simple and reproducible genetic transformation protocol for generating transgenic petunia plants harboring a gene of interest and selectable marker. The system utilizes *Agrobacterium tumefaciens* for transgene integration with plant recovery via shoot organogenesis from leaf explant material. Selection for transgenic plants is achieved using the *bar* gene conferring resistance to glufosinate or *npt*II gene for resistance to kanamycin. Transformation efficiencies of around 10% are achievable with shoots being recovered about 8 wk after transgene insertion and rooted plants transferred to the greenhouse about twelve weeks after inoculation.

Key Words: Petunia; *Petunia hybrida*; transformation; *Agrobacterium tumefaciens*; glufosinate; leaf disk transformation.

1. Introduction

The major role of petunia transformation has been assisting researchers to increase understanding of plant biochemical pathways. Of these biochemical pathways, the most common application of petunia transformation has been researching the various genes responsible for production of anthocyanins *(**1**,**2**)*. Since anthocyanins play a major part in flower coloration, numerous genes in the phenylpropanoid pathway have been extensively studied. By overexpressing *(**1**)*, suppressing *(**2**)*, or silencing *(**3**)* genes in this pathway, researchers have greatly increased understanding for this important pathway and generated novel transgenic petunia plants with commercial value as ornamentals *(**4**)*. Petunia transformation has also allowed researchers to investigate expression of modified bacterial genes in plant cells *(**5**)*, stress response *(**6**)*, and reporter gene studies *(**7**)*.

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Petunia transformation has been utilized since the early 1980s when protoplasts were isolated, cultured, and transformed with *Agrobacterium tumefaciens*. Although whole transgenic plants were not recovered from these early studies (*8*), they did demonstrate the ability to incorporate recombinant DNA into plant cells and obtain resistance to various antibiotics (*5*). Having shown that plant cells can express transgenes, researchers began to explore novel areas in the field of biotechnology and genetic research. As research progressed, new methods of transformation were developed. Petunia leaf disk transformation and plant regeneration became a more suitable tool for research because of its simplicity, the ability to maintain transformed cells in culture, and, most importantly, to regenerate fertile plants harboring transgenes of interest (*9,10*). In this chapter a robust method for petunia genetic transformation and plant regeneration is described using in vitro leaf disk explants, and provides a simplified alternative to protocols described previously (*1,2,11*). By utilizing excised leaf pieces from in vitro grown seedlings, the risk of exogenous contamination is minimized and transformation efficiencies of approx 10% are obtained (expressed as the number of transgenic shoot starting number of explants). Variation in transformation efficiency may depend on petunia variety, selection regime employed, and the nature of the transgene cassettes inserted.

2. Materials

2.1. Plant Material and *Agrobacterium* Strains

1. Petunia seed varieties used: Mariner (grandiflora type) obtained from Germania Seed Company (Chicago, Illinois), wild-type white flower variety maintained inhouse (*see* **Note 1**).
2. *Agrobacterium* strains/vector: GV3101 (*3,6*) and C58C1 (*see* **Note 2**). The binary vector used in this protocol, is a derivative of pBIN19 vector harboring kanamycin for the plasmid selection. The binary vectors were introduced into the bacteria using the Bio-Rad *Escherichia coli* Pulser electroporation device.

2.2. Plant Hormones and Media Additives

1. 1 mg/mL 6-benzylaminopurine (BAP) (Sigma; cat. no. B3408) stock solution. Prepare stock by adding 50 mg BAP to a 50-mL centrifuge tube and add a few drops of 1*M* KOH until dissolved. Bring to volume to 50 mL with tissue culture (TC) water (MilliQ system water with a resistance of 18 mΩ and total organic carbon [TOC] < 5 ppb). Filter-sterilize (*see* **Note 3**), aliquot to sterile 15-mL centrifuge tubes, and store at −20°C for up to 6 mo until use.
2. 1 mg/mL α-naphthaleneacetic acid (NAA) (PhytoTechnology Laboratories cat. no. N600) stock solution: Prepare by adding 50 mg to a 50-mL centrifuge tube. Dissolve in a few drops of 1 *M* NaOH and bring to 50 mL with TC water. Filter-sterilize, aliquot to sterile 15-mL centrifuge tubes, and store at −20°C for up to 6 mo until use.

3. 1 mg/mL *p*-chlorophenoxyacetic acid (PCPA) (PhytoTechnology Laboratories; cat. no. C 213) stock solution:. Prepare by adding 50 mg to a 50-mL centrifuge tube. Add a few drops of 1 *M* KOH until it is dissolved. Bring to 50-mL with TC water, filter-sterilize, and aliquot to sterile a 15-mL centrifuge tubes and store at –20°C for up to 6 mo until use.
4. 0.2 *M* Acetosyringone (ACS) (4-hydroxy-3′,5′-dimethoxyacetophenone) (PhytoTechnology Laboratories, cat. no. A104) stock solution: Prepare by weighing 392.4 mg into a 15-mL centrifuge tube. Dissolve in approx 7 mL of 100% ethanol. Bring to volume with 100% ethanol to reach 10 mL. Filter-sterilize and make 1 mL aliquots in sterile 1.5-mL tubes and store at –20°C for up to 6 mo.

2.3. Selective Agents and Antimicrobials

1. 10 mg/mL Glufosinate ammonium (Sigma, cat. no. 45520; CAS no. 77182-82-2) (*see* **Note 4)** stock solution: Prepare by adding 100 mg to a 10-mL centrifuge tube, dissolve, and bring to 10 mL volume in TC water. Stock is filter sterilized and stored at –20°C for up to 2 mo until use.
2. 250 mg/mL Carbenicillin (Carb) (PhytoTechnology Laboratories; cat. no. C346) stock solution: Prepare by adding 2.5 g to a 15-mL centrifuge tube. Add approx 5 mL TC water, vortex to dissolve, bring to 10-mL volume, filter-sterilize, and store at –20°C for up to 6 mo.
3. 100 mg/mL Kanamycin monosulfate (KAN) (PhytoTechnology Laboratories; cat. no. K 378) (*see* **Note 4)** stock solution: Prepare by adding 5 g to a 50-mL culture tube and adding approx 25 mL TC water until KAN is dissolved. Bring to 50-mL volume with TC water, filter-sterilize, and aliquot to 15-mL centrifuge tubes. Stocks are stored at –20°C for up to 6 mo.
4. 25 mg/mL Rifampicin (PhytoTechnology Laboratories; cat. no. R501) stock solution: Prepare by adding 250 mg to a 15-mL centrifuge tube and adding 5 mL of 100% ethanol. Vortex until dissolved and bring to final volume of 10 mL with 100% ethanol. Filter-sterilize stock solution to remove particles. Dispense 1-mL aliquots into 1.5-mL Eppendorf tubes and store at –20°C for up to 6 mo.

2.4. Plant Media

1. Murashige and Skoog (MS) Basal Medium: Prepare by adding 4.3 g MS basal salt mixture (PhytoTechnology Laboratories; cat. no. M524), 1 mL/L of Gamborg's vitamin solution 1000X (PhytoTechnology Laboratories; cat. no. G219) and 30 g sucrose to 800 mL of TC water (*see* **Subheading 2.2.1.**). Bring volume to 1000 mL with TC water. Adjust pH to 5.7 with 1*M* KOH, add 9 g TC agar (PhytoTechnology Laboratories; cat. no. A175) and autoclave at 121°C for 20 min.
2. Germination Medium: MS basal medium prepared and poured into 100 × 25-mm plates. One liter generates 28 to 30 plates.
3. Plant maintenance medium: MS basal medium prepared and poured into plastic sundae cups (Renard Paper Bottoms, cat. no. SO-8 [Tops], cat. no. SDL 58 [Bottoms]), each liter generates 25 to 28 sundae cups (*see* **Note 5**).

4. Liquid preculture medium: Prepare by adding 4.3 g MS basal salt mixture, 1 mL/L of Gamborg's vitamin solution 1000X, 30 g sucrose, and 4 mL PCPA stock solution to 800 mL of TC water. Bring volume to 1000 mL with TC water. Adjust pH to 5.7 with 1 *M* KOH. Pour 500 mL into a 1-L glass Schott bottles and autoclave at 121°C for 20 min (*see* **Note 6**).
5. Co-culture Medium: Prepare by adding 4.3 g MS basal salt mixture, 1 mL/L of Gamborg's vitamin solution 1000X 30 g sucrose, 1 mL of BAP stock solution, and 0.1 mL of NAA stock solution to 800 mL of water. Bring volume to 1000 mL with MilliQ water (*see* **Note 7**). Adjust pH to 5.7 with 1 *M* KOH add 9 g TC agar; autoclave at 121°C for 20 min. When the media cools to between 55 and 60°C pour into 100 × 15-mm culture plates. About 50 plates can be poured from 1 L.
6. *Agrobacterium* wash solution: $^1/_4$ strength MS medium. Prepare by adding 1.1 g MS basal salt mixture to 800 mL TC water (*see* **Note 6**) and bring to a final volume of 1000 mL. Adjust pH to 5.8 with 1*M* KOH and autoclave at 121°C for 20 min. Let media cool to room temperature, or store at 4°C until needed. At time of use add 2 mL of carbenicillin stock solution per liter of $^1/_4$ MS liquid wash solution.
7. Selection medium: MS Basal Medium with BAP 1 mg/L, NAA 0.1mg/L, glufosinate 15 mg/L, and carbenicillin 500 mg/L media. Prepare by adding 4.3 g MS basal salt mixture, 1 mL/L of Gamborg's vitamin solution 1000X, 30 g sucrose, 1 mL of BAP stock solution, and 0.1 mL NAA stock solution to 800 mL of TC water. Bring to a volume of 1000 mL with TC water, adjust pH to 5.7 with 1 *M* KOH, and add 9 g/L TC agar and autoclave at 121°C for 20 min. When media cools to between 55 and 60°C, add 2 mL of carbenicillin stock solution and 1.5 mL of glufosinate stock solution (*see* **Note 4**). Pour media into 100 × 25-mm plates to generate 28 to 30/L.
8. Rooting and seedling screening medium: MS basal medium supplemented with glufosinate 10 mg/L and carbenicillin 500 mg/L. Prepare by adding 4.3 g MS basal salt mixture, 1 mL/L of Gamborg's vitamin solution 1000X, 30 g sucrose to 800 mL of TC water. Bring to a volume of 1000 mL with TC water, adjust pH to 5.7 with 1 *M* KOH, and add 9 g/L TC agar and autoclave at 121°C for 20 min. When media cools to between 55 and 60°C add 2 mL of carbenicillin stock solution and 1 mL glufosinate stock solution (*see* **Note 4**). Pour media into 100 × 25-mm plates to generate 28 to 30/L.

2.5. Agrobacterium *Culture Media*

1. Growth medium (liquid): Luria-Bertani (LB) liquid medium prepared by adding 12.5 g (25 g/L) LB powder (PhytoTechnology Laboratories; cat. no. L475) to 400 mL TC water. Dissolve completely and bring to a volume of 500 mL with TC water. Autoclave media (*see* **Note 6**) for 20 min at 121°C. Cool and store at room temperature.
2. Growth medium (solid): Prepare LB liquid medium as in 1 above. Add 7.5 g (15 g/L) bacteriological agar (PhytoTechnology Laboratories; cat. no. A296). Autoclave for 20 min at 121°C. Cool and store in bottles or pour into Petri dishes while still in liquid form (*see* **Note 9**).
3. Coculture medium: Preculture liquid media with acetosyringone (ACS) 200 µ*M*. Prepare preculture media as above media as described in **Subheading 2.3.4.**

above. Allow media to cool at room temperature or store at 4°C until needed. At time of use add 1 mL/L of ACS stock solution (*see* **Note 10**).

2.6. Other Supplies

1. Schott bottles (Fisher Scientific NC; cat. no. 9604720).
2. Nylon syringe filters 0.22 μm pore size (Fisher Scientific; cat. no. 09-719-C).
3. Sterile 1.5-mL Eppendorf tubes (Fisher Scientific cat. no. 05-406-16).
4. Filter paper: 7 cm grade 1 qualitative filter paper (Fisher Scientific; cat. no. 09-805C).
5. Vented tape: 3M Micropore Brand Tape (Fisher Scientific; cat. no. 19-027-761).
6. Potting mix: Sun Gro Metro Mix 360: 45 to 55 % Vermiculite, sphagnum peat, bark ash, limestone and wetting agent (Hummerts, St Louis, MO).

3. Methods

3.1. Explant Sterilization and Germination

1. Pour petunia seeds into a 1.5-mL sterile tube up to the 100 μL mark on the side of the tube. Add 1 mL of 70 % ethanol and invert tube several times to completely immerse seeds.
2. Spin tubes in a microcentrifuge for 30 s to bring all the seed off the walls of the tube and leave for 2 min (*see* **Note 11)**.
3. Remove ethanol with a 1-mL pipet and discard.
4. Prepare a 20% (v/v) bleach solution containing 0.05% (v/v) Triton-X 100 as a surfactant and add 1 mL to each tube. Invert tubes several times and then spin in a microcentrifuge for 30 s (*see* **Note 11**). Leave seeds submersed in bleach solution for 20 min at room temperature.
5. Remove bleach solution by pipetting with a 1 mL pipettor and discard (*see* **Note 12**). To each tube of seed, add 1 mL sterile TC water and invert several times. Spin the seeds down off the wall (*see* **Note 11**) and withdraw the water as above. Repeat twice for a total of three washes with sterile TC water.
6. Remove seed by first vortexing the seed to resuspension, then empty tube onto a 7-cm sterile filter paper, trying to spread seeds as evenly as possible. Dry seeds for approx 1 h in the laminar flow hood.
7. After the seed has dried (*see* **Note 13**), using sterile forceps grab the filter paper and shake the seed off evenly onto germination media at a rate of 30 to 50 seeds/plate.
8. Seal plates with parafilm and culture at 24°C with a 16:8-h photoperiod and a light intensity of 110 $\mu mol/m^2/sec$ for 2 wk (*see* **Note 14**).
9. Using forceps, transfer germinated seedlings to maintenance media at three plants per sundae cup ensuring that the root system is intact and within the media. Culture at 24°C with a 16:8 hr and light intensity at 110 $\mu mol/m^2/s$ for 3 to 4 wk.

3.2. Explant Preculture

1. After 3 to 4 wk growth in sundae cups (*see* **Note 15**), cut leaves from the upper half of the plant (leaves are typically about 2-cm wide and 3–4 cm long) using a no. 10 scalpel blade and to a sterile 100 × 15-mm petri plate.

2. Prepare explants by trimming away the edges of the leaf (about 2 mm). Make horizontal slices through the leaf to create explants approx 4 × 4 mm in size with cut surfaces on all 4 sides.
3. Transfer explants to plates containing co-culture media overlaid with sterile 7-cm filter paper, ensuring that the lower epidermis is facing up and the upper epidermis is in contact with the filter paper. A total of 30 to 40 leaf pieces are prepared on each plate.
4. After all explants are placed on co-culture plates, apply 2 mL of pre-culture liquid media to each plate (*see* **Note 16**), wrap with parafilm and culture at 24°C with 16:8-photoperiod and light intensity 110 μmol/m^2/s for 2 days.

3.3. Agrobacterium *Culture Preparation*

1. Two days before inoculation, *A. tumefaciens* strains (*see* **Note 2**) carrying binary vector of choice are initiated by taking a 10 μL loop and inserting it into a tube of frozen glycerol stock. The loop is transferred to a 3 mL culture of LB media with antibiotics appropriate for the *Agrobacterium* strain and plasmid combination (*see* **Note 17**). Cultures are grown by agitation at 200 rpm on an orbital shaker overnight at 28°C.
2. The next day, 100 μL of the culture is transferred to 25 mL LB media with the appropriate antibiotics and grown overnight on the shaker at 28 to 30°C.
3. On the day of transformation, the 25 mL *Agrobacterium* suspension is transferred to a sterile 50-mL culture tube and centrifuged at 4000*g* for 10 min to pellet the bacteria.
4. The supernatant is decanted and the pellet resuspended by vortexing in 25 mL of liquid preculture media. The OD_{600} is measured and the culture is further diluted with liquid coculture media to obtain an OD_{600} 0.4 to 0.5. This is typically a 1:5 dilution of the original culture.

3.4. Infection and Co-cultivation

1. After 2 d preculture, culture dishes containing the petunia leaf explants are removed from the growth chamber. Inoculation is achieved by pipetting 3 mL of *Agrobacterium* suspended in coculture media onto each plate and culturing at room temperature for 20 min with gentle swirling every 5 min. Several plates can be inoculated at a time, with 200 to 300 explants (7–8 plates) per construct.
2. After 20 min the excess *Agrobacterium* suspension is removed by tilting the plates at a 45° angle and pipetting away all liquid (*see* **Note 18**). A sterile 7-cm filter disk is gently placed on top of the explants to remove excess *Agrobacterium* from the explants.
3. Plates are wrapped with parafilm and co-cultured in the light room at 24°C and a photoperiod of 18:6 and a light intensity of 110 μmol/m^2/s for 3 d.
4. After coculture, explants are washed to remove residual *Agrobacterium* by adding 5 mL of *Agrobacterium* wash solution to each plate and leaving for 15 min at room temperature with gently swirling twice during the this period.
5. After the wash period, leaf explants are individually transferred to sterile 7-cm filter paper using forceps and blotted dry before placing on the selection media.

3.5. Selection and Regeneration

1. Blotted explants are transferred to selection media with the upper epidermis slightly pushed into the media to ensure good contact. About 15 leaf pieces are placed in this manner on each plate, wrapped with Micropore vented tape and placed in the 24°C light room with a photoperiod of 18:6 and a light intensity of 110 μmol/m^2/s and cultured for 1 mo.
2. After 1 mo in culture, explants are transferred to fresh plates of selection medium. At this time green resistant callus on the leaf explants can be observed. The leaf pieces and callus are not deliberately separated at this stage but subcultured intact to the new selection medium (*see* **Note 19**). Plates are wrapped with Micropore vented tape and cultured for 2 more wk in the 24°C light room with a photoperiod of 18:6 and a light intensity of 110 μmol/m^2/s.
3. After 6 wk on selection media, larger green callus, and early stages of shoot regeneration should be observed. Green callus is excised with forceps by gently pulling it away from the rest of the leaf material, and transferred to fresh plates of selection medium at about 20 calli/plate depending on the callus size. The green callus is transferred biweekly onto fresh media of the same type. The callus and associated regenerating shoots are cultured in the 24°C light room with a photoperiod of 18:6 and a light intensity of 110 μmol/m^2/s.
4. After 6 to 8 wk culture on selection medium, shoots will start to emerge from the callus. When regenerating shoots are 0.5 to 1 cm in size, they are isolated using sterile forceps and a no. 11 sterile scalpel blade. The shoots are excised at their junction with the callus to ensure that the meristem region is intact and cut surface is inserted about 2 mm deep into rooting medium (*see* **Note 20**). Ten to fifteen shoots can be transferred and cultured of each plate of rooting medium in this manner. Plates are wrapped in venting tape, and cultured at 24°C with a photoperiod of 18:6 in the 24°C light room with a light intensity of 110 μmol/m^2/s.

3.6. Transplanting to Soil and Growth Chamber Care

1. Putatively transgenic shoots will generate root systems after 2 to 3 wk on rooting medium and after approx 1 mo should have established a large enough root system to be transferred to soil (*see* **Note 21**).
2. Four inch (9.5 cm) pots are filled with Sun Gro MetroMix 360 potting mix and watered thoroughly.
3. Rooted shoots are gently pulled from the plates and excess agar removed using non-sterile TC or DI water. This is accomplished by placing the plantlets under the TC system water and gently squeezing the agar away from the roots with fingers.
4. A 2-cm diameter hole is made in the potting mix using a finger tip or marker and the rooted shoots placed in the hole and covered with the potting mix. The plantlets are then watered again. A sundae cup top (Renard Paper Tops SDL 58) is placed over the plantlet to maintain humidity and the plantlets transferred to the growth chamber and grown at 23°C with a photoperiod of 12:12, and light intensity of 200 μmol/m^2/s. The plants are maintained in these conditions until the next set of leaves are formed.

5. When new leaves are observed, the sundae cup lids are tilted to allow acclimatization to the new environment. After a further 3–4 d the cups can be removed completely.
6. Plants are grown for a further 2 wk in the growth chamber and then transferred to the greenhouse (*see* **Note 22**). When flowering commences, about 6 wk after transferring to soil, bags are placed over them until seed has been formed and matured. The bag is used to collect the seed.

3.7. Harvest and Progeny Screening

1. After about 10 wk from transplanting to soil, the seed pods can be harvested and dried out on the bench (*see* **Note 23**).
2. When the seed pods are crushed the seeds are released. The seed is then collected and sterilized using the same technique as above (*see* **Subheading 3.1.**).
3. The seed is germinated on rooting medium with glufosinate selection at 10 mg/L. After 7 to 10 d, effects of the selection can be observed by the resistant plantlets remaining are green and growing in a robust manner, while the nontransformed plantlets become yellow with little or weak growth.
4. Resistant seedlings are then transferred to soil (*see* **Subheading 3.6.**) after 2 to 3 wk and the seed is collected when the plant matures as above (*see* **Subheading 3.7.**).

4. Notes

1. The white flower variety originated from a pack of mixed commercial seed and was chosen due to its floral organ phenotype. This is a self -pollinating variety currently being maintained in the Donald Danforth Plant Science Center's greenhouse for research purposes. Seeds are available upon request.
2. Details of the protocol described here have been developed for use with *Agrobacterium tumefaciens* strain C58C1. GV3101 has also been tested extensively and in our hands performs comparably to C58C1. Use of more virulent strains, for example LBA4404, have been reported *(1,7)* but some details such as co-culture times and antibiotics may need to be adjusted from the protocol described above if optimal transformation efficiencies are to be obtained.
3. Filter-sterilize all additives unless specified, using a sterile nylon syringe filter unit with a pore size of 0.22 µm.
4. Glufosinate is the preferred selection method for this protocol, but kanamycin can be used at 300 mg/L for the callus induction/selection and plant regeneration phases and at 100 mg/L for the rooting process.
5. Single-use sundae cups are not guaranteed sterile but are food service quality clean. Make sure the bags/wrappers around the stack of cups and bottoms is not torn, ripped, or punctured by objects. If desired, use autoclavable, re-useable Magenta boxes, (PhytoTechnology Laboratories; cat. no. C906) as an effective alternative.
6. Schott bottles are used for preparation of all liquid media. Liquid media is poured into bottles until 50 to 75% full, depending on the quantity of media to be made. This ensures that media does not boil out during autoclaving.

7. Growth regulators are added to MS basal media before the pH step. With the addition of BAP, the pH of the media will increase. If the reader wishes to add BAP and NAA after autoclaving, it is recommended to include a buffer such as MES [(2-(N-Morpholino) ethanesulfonic acid) PhytoTechnology Laboratories; cat. no. M825] in the media to produce a concentration of to 10 to 20 m*M* prior to adjusting the pH and autoclaving. Another alternative is to make a concentrated stock (2–3 mg/mL) of BAP and add smaller amounts to the post autoclaved media. Typically this will increase the pH by less than 0.05.
8. If bacterial contamination is present during the screening process it may be necessary to add carbenicillin at 500 mg/L.
9. Sterilized LB agar can be stored at room temperature in solid form in bottles until needed. LB agar is then melted in the microwave on defrost cycle (250 mL requires about 10 min at this setting) and the appropriate selection agents added once the temperature has cooled to between 50 to 60°C. Media is poured into 100 × 15 mm plates.
10. When coculture media is needed, typically 250 to 500 mL of media is used at a time and the appropriate amount of ACS stock added immediately prior to use.
11. Speed of the seed spin is not of critical importance as the aim is to bring all seed to the bottom of the tube for complete submersion.
12. Be careful to not pipet seeds up into the pipet tip. This can be accomplished by withdrawing the bleach solution from the middle of the tube until the solution reaches the seeds. The tip is then pushed down to the very bottom of the tube and the remaining liquid extracted.
13. Seed is considered dry when the filter paper has dried out and the seeds roll around freely. If some seed remains stuck to the paper, they can be removed by gentle scraping with a sterile scalpel blade.
14. Isolated bacterial colonies can be removed if needed from the medium with a sterile scoop. If fungal contamination is observed, such plates should be discarded.
15. Explants taken at this time exhibit bigger leaves. An alternative the protocol is to leave the seedlings on the germination plates for one month without transferring. These leaves are then cut directly from the germination plate and used for *Agrobacterium* inoculation.
16. When adding preculture media to the surface of the coculture plates, be careful not to cause the explants to float on top of the liquid media. This depends mainly on the absorbency of the filter disk. This protocol uses 7-cm grade 1 qualitative filter paper autoclaved for 30 min on a dry cycle. Nine-centimeter filter paper (Fisher Scientific; cat. no. 09-805D) can be used, but is harder to place on the media . If 9-cm filter papers are used, gently push all edges so the paper is somewhat flat against the media. If explants do float, then remove excess fluid with a 1-mL pipet so that the explants make contact with the filter paper, while ensuring that the filter paper remains fully saturated.
17. Typical *Agrobacterium* selection is rifampicin at 30 mg/L. Selection for presence of the desired plasmid varies and should be ascertained from its source, prior to initiation of the transformation process.

18. At this step it is important that almost all *Agrobacterium* suspension is removed. If excess *Agrobacterium* is not removed, spill-over is possible and contamination or *Agrobacterium* cross-contamination may occur. The plates are tilted against a 1.5-mL centrifuge tube rack, which provides the angle necessary to drain excess fluid and allow its removal with a sterile pipet. If explants become detached from the filter paper, they can be replaced using sterile forceps.
19. At time of subculture the callus may break away from the rest of the leaf material. In this case the callus is transferred to the new medium along with the remaining leaf piece. If more than one callus clump is present on a leaf but they are clearly separated from each other, they can be considered the result of independent transformation events and may be treated as distinct individuFals. At this time the callus ranges from 1 mm to 1 cm in size, but the larger the callus, the better the chance of recovering shoots. If the plates appear to be drying out before the stipulated 1-mo culture period, then the callus/leaf pieces can transferred to new selection plates as needed. The vented tape allows for more air exchange than parafilm, and as a result the plates may dry out quicker, depending on airflow in the grow rooms.
20. If more than one shoot is recovered from the same callus, such shoots should be isolated but placed adjacent to each other on the rooting media and marked accordingly. For example, a circle may be drawn on the plate bottom to indicate that these multiple shoots originated from the same callus and are considered siblings, or clones, recovered from the same transgene integration event. Recovering multiple shoots in this manner is recommended to ensure that at least one transgenic event survives for further study.
21. If shoots have not produced roots but remain green as opposed to yellow, the base of the shoot is cut and the remaining shoot is transferred to new rooting media. At times, the callus at the base of the shoot does not differentiate to form roots. It may be removed if necessary.
22. Greenhouse conditions vary to some extent depending on the season and quality of supplemental lighting and temperature/humidity control. A temperature of 24°C, light intensity about 500 µmol/m^2/s, and a photoperiod of 14:10 are the conditions favorable for obtaining seed from petunia.
23. The flowering and seed collection times can vary according to the greenhouse conditions and the transgenic lines. Generally, mature seed can be collected about 8 wk after transferring plantlets to the greenhouse.

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Rose (*Rosa hybrida* L.)

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Summary

Although rose transformation is successful, it remains difficult to transform myriad rose species as well as different rose genotypes. In this protocol, a detailed description of rose transformation is presented. This protocol relied on *Agrobacterium*-mediated transfer of embryogenic callus cultures. There are many critical steps that must be followed to achieve successful transformation; however, it is important to keep in mind that these apply to a selected number of genotypes, and as a different genotype is subjected to transformation, modifications of this protocol must be made to achieve successful transformation.

Key Words: *Agrobacterium tumefaciens*; *Rosa hybrida*; genetic transformation.

1. Introduction

The high commercial value and widespread cultivation have made roses (*Rosa*) an important commodity of ornamental crops, and they have long been targeted for genetic improvement for myriad traits. Traditionally, rose improvement has depended on sexual hybridization and selection, together with identification of natural mutations. While these traditional approaches continue to be used in various breeding programs, they are ultimately restricted by heterozygosity and complex ploidy levels that are prevalent within this genus. Although the haploid chromosome number of rose is $x = 7$, only about half of the known rose species are diploid ($2x = 14$), while the remaining are polyploids, ranging from $4x = 28$ to $8x = 56$ ***(1)***. As segregation patterns for traits are more complicated in polyploids, this contributes to the cumbersome and difficult nature of genetic analysis in rose ***(2–3)***. Moreover, this allows for a limited gene pool for transfer of desirable horticultural traits ***(2–3)***. Therefore, genetic engineering strategies are highly desirable for genetic improvement of roses as they facilitate the introduction and/or modification of desirable traits without altering the overall quality of the

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target variety. Although regeneration has been reported for several species of rose—including *Rosa hybrida* (*4–9*), *R. rugosa* (*10*), *R. chinensis minima* (*7,9*), *R. canina* (*11*)—using various vegetative and reproductive tissues as explants, there are only a handful of successful reports on rose transformation (*12–19*).

Both particle bombardment- and *Agrobacterium*-mediated methods have been successfully used for genetic transformation of rose (*12,15,17*). Nevertheless, both protocols have been limited to selected genotypes of rose. Because of this high genotypic influence on successful transformation of rose, additional modifications of current protocols must be made whenever a different rose genotype, other than the one used here, is subjected to genetic engineering efforts.

In this proposed protocol, we will provide a detailed description of the protocol used in our laboratory for *Agrobacterium*-mediated transformation of embryogenic cultures of rose using *R. hybrida* cv. Carefree Beauty. The transformation efficiency, based on number of confirmed transgenic plants (following Southern blotting) per explant (primary embryogenic callus), is calculated at 10%.

2. Materials

2.1. Agrobacterium *tumefaciens Strain*

Agrobacterium tumefaciens strain GV3101, harboring the plasmid pCAMBIA 2301 (CAMBIA, Canberra, Australia) or any derivative of this binary vector, is used. This plasmid carries the *uidA* reporter gene that codes for β-glucuronidase (GUS) as well as the *npt*II selectable marker gene that codes for neomycin phosphotransferase II.

2.2. Culture Media for Agrobacterium tumefaciens

1. Luria–Bertani (LB) medium: 20 g/L LB broth base (Invitrogen, Carlsbad, CA), 15 g/L Bacto agar (Invitrogen). Autoclave for 15 min at 121°C, then cool down to 55°C prior to adding the selective agent, depending on the selectable marker gene. Use 1 mL liquid medium.
2. YEP medium: 10 g/L Bacto-peptone, 10 g/L Bacto-yeast extract, 10 g/L NaCl. Autoclave for 15 min at 121°C, then cool down to 55°C prior to adding the selective agent, depending on the selectable marker gene. Use 50 mL liquid medium containing 100 mg/L kanamycin.

2.3. Plant Material

Embryogenic callus, induced from in vitro-grown leaflet tissue of cv. Carefree Beauty, is used for *Agrobacterium*-mediated transformation.

2.4. Media Stock Solutions

1. All stocks are made with distilled-deionized water (ddH_2O), and stored at 4°C unless otherwise noted.

2. All antibiotic solutions were filter-sterilized and added to media after autoclaving.
3. 5% Hypochlorite solution.
4. Murashige and Skoog (MS) basal salt mixture (PhytoTechnology Laboratories, Shawnee Mission, KS).
5. 1000X MS vitamin powder (PhytoTechnology Laboratories, Shawnee Mission, KS): Dissolve 10.31 g of MS vitamins in 100 mL ddH_2O. Store at 4°C for up to 6 mo. Add 1 mL/L to medium before autoclaving.
6. 2 mg/mL stock 6-Benzyladenine (BA) (SIGMA, St. Louis, MO): Dissolve 20 mg BA in 2 mL 1 *N* NaOH in a beaker with a magnetic stir bar. Bring final volume to 10 mL with ddH_2O. Store at 4°C for up to 6 mo.
7. 1 mg/mL Naphthalene acetic acid (NAA) (SIGMA): Dissolve 10 mg NAA in 2 mL 1 *N* NaOH in a beaker with a magnetic stir bar. Bring final volume to 10 mL with ddH_2O. Store at 4°C for up to 6 mo.
8. 1 mg/mL 2,4-Dichlorophenoxyacetic acid (2,4-D) (Sigma): Dissolve the powder in a few drops of 1*M* KOH then add ddH_2O to volume. Store at 4°C up to 3 mo.
9. 1 mg/mL Thidiazuron (TDZ) (AgrEvo, USA Company): In hood, dissolve 10 mg TDZ in 2 mL dimethyl sulfoxide (DMSO) (Fisher Scientific) in a beaker with a magnetic stir bar. Bring final volume to 10 mL with ddH_2O. Store at 4°C for up to 6 mo.
10. 1 mg/mL Gibberellic acid (GA_3) (Phytotechnology Laboratories): Dissolve 10 mg GA_3 in 2 mL absolute alcohol in a beaker with a magnetic stir bar. Bring final volume to 10 mL with ddH_2O. Store at 4°C for up to 6 mo.
11. 100 mg/mL Kanamycin monosulfate (PhytoTechnology Laboratories): Dissolve 1 g kanamycin monosulfate in 10 mL ddH_2O in a beaker with a magnetic stir bar. Filter-sterilize, and divide into 1 mL aliquots. Store at –20°C for up to 6 mo.
12. 500 mg/mL Cefotaxime sodium salt (Sigma): Dissolve 2 g cefotaxime sodium salt in 10 mL ddH_2O in a beaker with a magnetic stir bar. Filter-sterilize and divide into 1-mL aliquots. Store at –20°C for up to 6 mo.
13. Soil mix: Sunshine Mix no.1, Sun Gro Horticulture Inc.

2.5. Tissue Culture Media

1. Shoot proliferation medium: Full-strength MS salts and vitamins supplemented with 0.5 mg/L BA, 0.05 mg/L NAA, 30 g/L sucrose, 2.5 g/L gelrite gellan gum (Sigma); adjust pH to 5.7, autoclave for 20 min at 121°C, and pour into glass jars (50 × 85 mm) (*see* **Note 1**).
2. Callus induction medium: Full-strength MS salts and vitamins supplemented with 2.5 mg/L 2,4-D, 30 g/L sucrose, 2.5 g/L gelrite gellan gum; adjust pH to 5.7, autoclave for 20 min at 121°C, and pour into 100 × 15-mm Petri plates.
3. Embryogenic callus medium A: Half-strength MS salts and full-strength MS vitamins supplemented with 0.5 mg/L TDZ, 1.0 mg/L GA_3, 30 g/L sucrose, 2.5 g/L gelrite gellan gum; adjust pH to 5.7, autoclave for 20 min at 121°C, and pour into 100 × 15-mm Petri plates.
4. Embryogenic callus medium B [plant-growth regulator (PGR)-free medium]: Half-strength MS salts and full-strength MS vitamins, 30 g/L sucrose, 2.5 g/L

gelrite gellan gum, adjust pH to 5.7, autoclave for 20 min at 121°C, and pour into 100 × 15-mm Petri plates.

5. Cocultivation medium: Half-strength MS salts and full-strength MS vitamins, 30 g/L sucrose, and 2.5 g/L gelrite gellan gum, pH 5.7.
6. Selection medium A: Half-strength MS salts and full-strength MS vitamins, 10 g/L sucrose, 2.5 g/L gelrite gellan gum, pH 5.7, 500 mg/L cefotaxime, and 100 mg/L kanamycin (*see* **Notes 2** and **3**).
7. Selection medium B: Half-strength MS salts and full-strength MS vitamins, 10 g/L sucrose, 2.5 g/L gelrite gellan gum, pH 5.7, 250 mg/L cefotaxime, and 100 mg/L kanamycin (*see* **Notes 2** and **3**).
8. Shoot regeneration medium: Half-strength MS salts and full-strength MS vitamins, 10 g/L sucrose, 0.5 mg/L BA, and 2.5 g/L gelrite gellan gum, pH 5.7.
9. Rooting medium: Full-strength MS salts and MS vitamins, 30 g/L sucrose, and 2.5 g/L gelrite gellan gum, pH 5.7.
10. Liquid MS medium: Half-strength MS dissolved in ddH_2O, and autoclaved for 20 min at 121°C.

3. Methods

3.1. Plant Material Preparation (20)

3.1.1. Initiation and Proliferation of Shoot Culture

1. Nodal stem segments (1.5–2.0 cm in length) are collected from current season growth of greenhouse-grown plants. These are surface-sterilized with 0.525% sodium hypochlorite (10% Clorox® commercial bleach) for 10 min, and rinsed three times with ddH_2O.
2. Nodal stem explants are placed in glass jars, 4 to 5 explants/jar, containing 25 mL of shoot proliferation medium. Cultures are grown in a growth chamber under a 16-h photoperiod provided by cool-white 40-watt fluorescent tubes (60 $\mu mol/m^2/s$) at 24±1°C.
3. Shoot cultures should be transferred to fresh medium once every 4 to 5 wk. It will take numerous subcultures, over a period of 6 to 7 mo, before there is enough leaf tissue to proceed with inducing embryogenic callus cultures.

3.1.2. Initiation of Callus Culture (9)

1. The top four vigorously-growing leaves are excised from in vitro-grown proliferating shoots. Either whole leaves (*see* **Fig. 1A**) or leaflets can be used as explants for callus induction *(7,9)*. All leaf explants should be placed with the abaxial surface in contact with the callus induction medium (25 mL/plate) in 100 × 15-mm Petri plates. Use approx 10 to 15 explants per plate. Cultures are then incubated in the dark for 4 wk at a temperature of 23±1°C. Approximately 25% of explants will develop embryogenic callus, whereas another 10 to 30% of explants will develop either organogenic callus or undifferentiated callus.
2. Explants with callus are then transferred to 100 × 15-mm Petri plates containing embryogenic callus induction medium A (*see* **Fig. 1B**). Cultures are grown in a

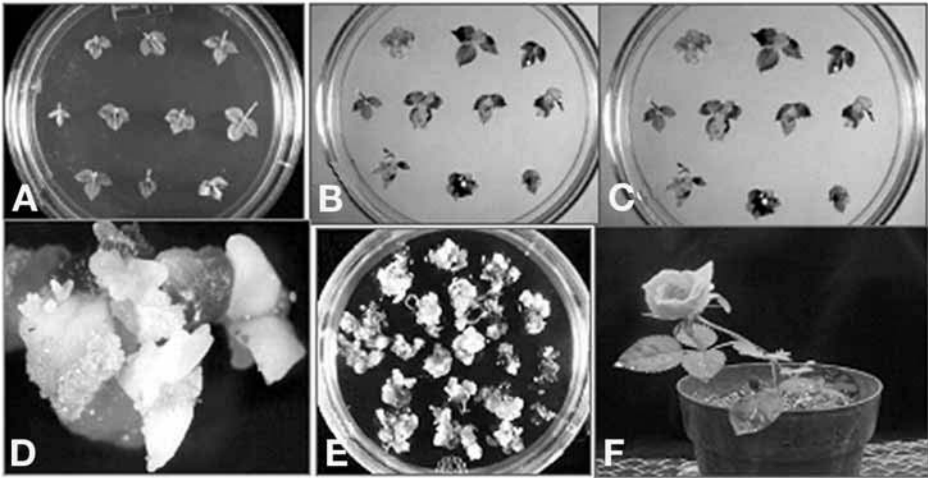

Fig. 1. *Agrobacterium*-mediated transformation of *Rosa hybrida* L. cv. Carefree Beauty. (**A**) Leaf explants in Petri dishes. (**B**) Callus induction. (**C**) Embryogenic callus induction *Agrobacterium* inoculation. (**D**) Primary somatic embryos following cocultivation of embryogenic cultures with *Agrobacterium tumefaciens*. (**E**) Shoot regeneration of putative transgenic lines: (**F**) Flowering of well-developed transgenic plantlets in greenhouse.

growth chamber under a 16 h photoperiod provided by cool-white 40-watt fluorescent tubes (60 µmol/m^2/s) at 24±1°C. Cultures are grown for a period of 8 wk. Explants are then subcultured to embryogenic callus induction medium B for an additional 8 wk (*see* **Fig. 1C**).

3.2. Agrobacterium *tumefaciens Preparation*

1. Streak *Agrobacterium* cells from a permanent glycerol stock onto solid LB with 100 mg/L kanamycin, and then incubate for about 2 d at 28°C or until colonies appear.
2. A single colony is transferred to 50 mL of liquid LB medium with 100 mg/L kanamycin, and grown overnight at 28°C under continuous shaking (175 rpm).
3. On the following day, 1 mL of overnight-grown culture is re-cultured in 50 mL liquid LB medium until an OD_{600} reading of between 1.3 and 1.5 is obtained.
4. Before preparing and inoculating explants, the bacterial culture is centrifuged at 6000*g* for 5 min at 24°C. The resulting pellet is resuspended in 50 mL liquid MS and divided into 25 mL aliquots.
5. Inoculum is prepared by adjusting bacterial suspension to OD_{600} = 0.2 with liquid MS (*14*).

3.3. Explant Preparation and Inoculation

1. All manipulations involving plant and bacterial materials are done in a laminar flow hood under sterile conditions.

2. Prepare embryogenic calli induced from in vitro-grown leaf tissues. Cut embryogenic callus into small pieces, injure tissue with a sterile needle, and immerse them into liquid MS until all explants are prepared (*see* **Note 4**).
3. When explants are prepared, replace liquid MS with the bacterial suspension (OD = 0.2), and inoculate for 30 min.
4. After immersion in the bacterial suspension, blot explants dry with sterile filter paper to remove excess liquid containing *Agrobacterium*, and place them on co-cultivation medium for 48 h at 25°C.

3.4. Selection and Plant Regeneration

1. After 2 d on co-cultivation medium, rinse explants with ddH_2O containing 250 mg/L cefotaxime, blot dry onto a sterilized filter paper, and then transfer to selection medium A. Explants are transferred to a fresh selection medium once every 2 wk for a period of 8 wk (*see* **Fig. 1D**). Throughout, explants were incubated under a 16-h photoperiod (25 µmol/m^2/s) at 25°C.
2. Transfer explants to fresh selection medium B, whereby the cefotaxime concentration in the selection medium is reduced to 250 mg/L. Explants are transferred to a fresh selection medium once every 3 to 4 wk (*see* **Note 5**).
3. Kanamycin-resistant embryogenic callus should be separated from darkened or bleached tissues during each subculture ***(14)***. Explants were incubated under a 16-h photoperiod (25 µmol/m^2/s) at 25°C.
4. After a period of 8 mo of selection, surviving embryogenic callus is transferred to shoot regeneration medium (*see* **Fig. 1E**). Shoot regeneration may take anywhere between 3 and 4 mo.
5. Transfer established shoots to rooting medium. After about 4 wk, plantlets (with well-established roots) are transferred to soil (*see* **Fig. 1F**). Almost 95% of shoots develop roots.
6. Leaf tissue will be collected from young plantlets and subjected to molecular analyses, including polymerase chain reaction (PCR) and Southern blotting to confirm presence and integration of the transferred gene.

3.5. Greenhouse Acclimatization and Care

1. When plantlets develop well-established roots, thoroughly wash them under tap water to remove residuals of medium, and then transfer them to soil in plastic flats (45 × 75 × 15 cm).
2. Flats with plantlets should be covered with either a clear plastic bag or a clear plastic covering, moved to the greenhouse, and grown at 25±1°C under 16-h photoperiod.
3. After 3 to 4 d, the plastic covering is gradually removed for the next 3 to 4 d to allow for acclimatization of plantlets. Almost 100% of rooted plantlets are successfully acclimatized with no problems.
4. Plants are watered on a daily basis and fertilized once a week with a standard Peter’s fertilizer (20:20:20 NPK).

5. Plants will usually flower within 6 to 9 mo following acclimatization (*see* **Fig. 1F**). Transgenic plants can be easily propagated via tissue culture (shoot proliferation) using protocols described above ***(7)***.

4. Notes

1. These glass jars can be either medium- or large-sized baby food jars, and closed with clear Magenta box lids (Magenta Corp, Chicago, IL).
2. All antibiotics are added to autoclaved media from previously prepared stocks.
3. The selectable marker gene used is *npt*II along with kanamycin at 100 mg/L as the selection agent for screening putative transformants. It is necessary to determine the critical level of the selectable agent in the selection medium that will only allow for survival of transformed plantlets ***(17,19)***.
4. Maintain explants liquid MS until ready for inoculation to prevent them from drying. Cutting embryogenic calli into smaller pieces, along with injuring callus, is very important to allow for good contact with the selection medium ***(17,18)***.
5. The development of embryogenic callus should be observed throughout this incubation regime. Embryogenic callus is soft, friable, and opaque-white in color. At times, explants might turn brownish in color (especially those continuously incubated on PGR-free medium), but this callus can still produce somatic embryos. However, if hard, compact, and green-colored callus is observed, it is most likely to be either a nondifferentiating callus or organogenic callus ***(19)***.

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VII

Medicinal Plants

33

Ginseng (*Panax ginseng*)

Yong Eui Choi

Summary

Genetic transformation is an attractive way to improve *Panax ginseng* C. A. Meyer by introducing new genetic materials and altering metabolic pathways that regulating the production of secondary compounds. In *P. ginseng*, production of transgenic plants via *Agrobacterium tumefaciens* is performed via somatic embryogenesis using cotyledon or embryogenic callus as explants. This chapter introduces the protocol of *A. tumefaciens* mediated-genetic transformation in *P. ginseng*.

Key Words: *Panax ginseng*; genetic transformation; *Agrobacterium tumefaciens*; somatic embryogenesis.

1. Introduction

Panax ginseng C. A. Meyer is a perennial herbaceous species, belonging to family Araliaceae. Roots of *P. ginseng*, one of the most famous and expensive crude drugs, is commonly used to promote quality of life *(1,2)*. Conventional breeding of *P. ginseng* is difficult because of un-conserved germplasm and long period (takes more than 50 yr). Genetic transformation is considered as an important approach for breeding of *P. ginseng*.

Genetic transformation of *P. ginseng* was tried extensively using *Agrobacterium rhizogenes* to induce hairy roots *(3)*. The first attempt for the production of transgenic ginseng plant using *Agrobacterium tumefaciens* was reported by Lee et al. *(4)*. They introduced the β-glucuronidase (GUS) gene into cotyledon explants by *A. tumefaciens* LBA4404 harboring pBI121, and the transgenic plants were obtained by somatic embryogenesis. Transgenic ginseng plants were produced from *A. rhizogenes*–transformed hairy roots via somatic embryogenesis, and the plants had actively growing root system similar to the characters of hairy roots *(5)*. A rapid and efficient genetic transformation of *P. ginseng* by

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plasmolyzing pre-treatment of cotyledon explants was reported (*6*). Cotyledon explants pre-treated with 1.0 *M* sucrose for 24 h showed enhanced both transient and stable expression of GUS, when co-cultivated with *A. tumefaciens* EHA101 harboring pIG121-Hm (*7*). Herbicide-resistant transgenic *P. ginseng* plants were produced by introducing the phosphinothricin acetyl transferase (PAT) gene through *Agrobacterium tumefaciens* co-cultivation (*8*). Temporary pre-treatment of embryogenic callus with 0.5 *M* sucrose for 3 h or 0.05 *M* $MgSO_4$ for 30 min enhanced the transient and stable expression of the GUS gene.

The genes involved in saponin biosynthesis are of considerable interest in the area of ginseng biotechnology. A more detailed understanding of these genes would facilitate the development of plants with altered or novel saponin content by transformation-mediated genetic modification. The roles of squalene synthase (PgSS1) protein in the biosynthesis of phytosterols and triterpenoids were investigated (*9*). Transgenic plants of *P. ginseng* expressing PgSS1 gene were produced successfully using the protocol as described by Choi et al. (*6*) and transgenic ginseng plants resulted in remarkable increase of phytosterol as well as triterpene saponin (ginsenoside) content (*9*). The present chapter describes the *Agrobacterium*-mediated transformation protocol that can be widely applied for laboratory and industrial scale.

2. Materials

2.1. Plant Material

Seeds of *P. ginseng* C. A. Meyer (*see* **Note 1**) containing mature zygotic embryos after moist-chilling treatment are prepared to dissect out the zygotic embryos. Cotyledon explants are used directly or by inducing embryogenic callus (*6,8*).

2.2. Agrobacterium *Strain and T-DNA Vectors*

1. *A. tumefaciens* GV3101 (*8*) or LBA4404 strain (*9*) is recommended for *P. ginseng* transformation experiments. However, there is no specific requirement for *Agrobacterium* strains.
2. Conventional binary vector derivatives including pBI and pCAMBIA series can be used for *P. ginseng* transformation experiments (*see* **Note 2**). There is no specific requirement for binary plasmids.

2.3. Media

1. The preparation of plant media for callus culture, somatic embryogenesis and plant conversion is described in **Table 1** (*see* **Note 3**). All media are adjusted to pH 5.8 before to being autoclaved at 120°C for 15 min.
2. Murashige and Skoog (MS) medium: We use the two kinds of commercial powder including MS salt and vitamin (Duchefa, The Netherlands; cat.no. M0222.0050),

Table 1
Medium for Somatic Embryo Induction and Plant Conversion in *Panax ginseng*

Step	Medium	Growth regulators	Carbohydrate	Geling agent
Embryogenic callus culture (EC)	MS	2,4-D (1 mg/L)	Sucrose (3%)	Agar (0.7%)
Somatic embryo induction (SI)	MS	Free	Sucrose (5%)	Agar (1%)
Embryo germination (EG)	$^1/_2$ MS	GA_3 (5 mg/L)	Sucrose (2%)	Gelrite (0.25%)
Plantlet growth (PG)	$^1/_2$ MS-NH_4NO_3	Free	Sucrose (2%)	Gelrite (0.25%)

Note: The pH of all media is adjusted to 5.8.

or MS basal salt mixture lacking NH_4NO_3 (Duchefa, The Netherlands; cat.no. M0238.0050).

3. Solidifying agent (*see* **Note 4**):
   a. Agar (Junsei Chemical Co., Japan): Used for the embryogenic callus and somatic embryo induction medium.
   b. Gelrite (Duchefa, Haarlem, The Netherlands): used for the germination and plant growth medium.
4. YEB *(12)*, (for *Agrobacterium* culture): 10 g/L of yeast extract, 10 g/L of peptone, and 5% of sucrose, pH 7.2. For solid medium, add 15 g/L Bacto agar before autoclave.

2.4. Stock Solutions

2.4.1. Phytohormone Stocks

1. 0.2 mg/mL 2,4-dichlorophenoxy acetic acid (2,4-D): (Stock solution in water.) Final concentration is 1 mg/L. First dissolved in a small volume of 100% ethyl alcohol and adjusted to 0.2 mg/mL with water.
2. 0.2 mg/mL Gibberellic acid (GA_3): Stock solution Dissolve in a small volume of ethanol and adjust to 0.2 mg/mL with water. Final concentration is 5 mg/L.

2.4.2. Antibiotics stocks (see **Notes 5** and **6**)

1. 0.2 g/mL Cefotaxime (Duchefa): (Stock in sterile water). Final concentration for media is 400 mg/L for first 3 subcultures and 200 mg/L thereafter.
2. 40 mg/mL Kanamycin (Duchefa, Haarlem, The Netherlands): (Stock in sterile water). Final concentration for media is 50 mg/L.
3. 50 mg/mL Hygromycin (Duchefa): Final concentration for media is 10 mg/L.

2.5. Other Supplies

1. 14-ml Polypropylene round-bottom tube (Falcon, Bection Dickinson Labware) for culturing of *Agrobacterium*.

2. Shaking incubator (100–200 rpm on a gyratory shaker).
3. 1.5-mL Eppendorf tube to spin down the *Agrobacterium* to exchange the YEB medium into callus culture liquid medium.
4. Centrifuge (3000*g*) for spin down the *Agrobacterium*.
5. Filter paper (Advantec, Toyo Rosgi Kaisha Ltd., Japan) for blotting to remove the remnant bacterial solution after dipping of explants in *Agrobacterium* solution (*see* **Note 5**).
6. Acetosyringone to stimulate the transformation frequency.
7. Parafilm.
8. Erlenmeyer flask (100–200 mL) for culture of plantlets.
9. Autoclavable polypropylene or polycarbonate large box (1 L) for aseptic culture of plants in perlite and peat moss mixture.
10. Sterilized perlite and peat moss for acclimatization of plants.
11. Culture room condition, where temperature, humidity, air exchange and illumination are automatically controlled.

3. Methods

Selection of transgenic callus is the most important point to produce transgenic plants (*see* **Note 6**), the protocol described below is for kanamycin selection.

3.1. Preparation of Plant Materials for Transformation

Cotyledon explants from zygotic embryos or embryogenic callus induced from cotyledons can be used for transformation experiments.

3.1.1. Preparation of Plant Materials From Sterilized Seeds (see **Note 7**)

1. Stratification (moist-chilling treatment for about 6 mo) of seeds is necessary because zygotic embryos in ginseng seeds are immature (globular to heart-shaped stage) after harvest (typical rudimentary embryos) ***(13)***. Moist treatment after mixing with sand for 3 mo (to support embryo growth until cotyledonary stage) and further chilling treatment (10°C) for 3 mo (to break the dormancy) ***(13)*** is necessary.
2. After removing the pericarp, seeds were immersed in 70% ethyl alcohol for 1 min and transferred to 1% sodium hypochloride solution for more than 20 min, and rinsed four times in sterile water.
3. Zygotic embryos were carefully dissected out from seeds and cultured on $^1/_2$ MS medium with 2% sucrose and solidified with 0.25% gelrite in 9-mm diameter Petri dishes, in the dark at room temperature.
4. Cotyledon explants before germination or within 3-d-old young seedlings are ready for transformation, or embryogenic callus induction.

3.1.2. Preparation of Embryogenic Callus (see **Note 8**)

1. Cotyledon explants are cultured on MS medium with 1.0 mg/L 2,4-D and 3.0 mg/L sucrose, solidified with 0.9% agar for about 3 d. Culture room was maintained at

24°C under a 16-h light/8-h dark photoperiod with light being supplied by white florescent tubes at an intensity of 24 µmol/m^2/s. Abaxial portion of cotyledon is placed on the medium surface which fosters the frequency of somatic embryo or embryogenic callus induction (*14*).

2. Embryogenic callus is mainly formed directly near the basal excised portion after 2 wk of culture. This embryogenic callus is selected from maternal cotyledon explants and sub-culture is done on the same medium as culture initiation at 1 mo intervals.
3. Agar is better than Gelrite not only for inducing embryogenic callus but also for maintaining embryogenic potential. Rapid sub-culturing at 2-wk intervals is not appropriate because embryogenic callus become friable and watery, and this callus is easy to lose embryogenic potential or hard to induce somatic embryos.

3.2. Preparation of Agrobacterium Culture

1. Two days before the transformation experiment, start an *Agrobacterium* liquid culture by inoculating a freshly grown single colony of *Agrobacterium* in 2 mL YEB liquid medium supplemented with the appropriate antibiotics for the selection of the transformation vector. The culture is incubated at 30°C (200 rpm), overnight in gyratory shaker.
2. The day before the transformation, a 14-mL polypropylene round-bottom Falcon tube containing 5 mL YEP medium (with appropriated antibiotics for selection) is inoculated with 100 µL *Agrobacterium* stock solution. This culture tube is incubated at 24°C over night (200 rpm). The OD_{600nm} of this culture should reach 0.6 on the day of the transformation.
3. Bacterial solution is divided into 1.5-mL Eppendorf tubes and centrifuged at 3000*g* for 10 min and re-suspended the pellet in sterile liquid medium for embryogenic callus culture (EC) with the same amount. The culture is then transferred into a Petri dish.

3.3. Explant Preparation (see Note 9)

Cotyledon explants or embryogenic callus are used for transformation. Friable embryogenic callus is not good because this callus is sensitive to *Agrobacterium*. Therefore, compact and nodular embryogenic callus or early developing somatic embryos (from globular to heart-shaped stage) is appropriate because this stage of explant has high frequency of somatic embryo formation.

1. Cotyledon explants or embryogenic callus without any pretreatment can be used for genetic transformation. However, plasmolyzing pretreatment of explants greatly enhances the transformation frequency (*6,8*).
2. Plasmolyzing pretreatment of explants enhances the frequency of not only transient GUS expression (*see* **Fig. 1A,B**) but also stable GUS expression (*6,8*). Cotyledons were pretreated in MS liquid medium containing 0.5 *M* or 1.0 *M* sucrose for 1 d. The pre-treated cotyledons were re-hydrated by half-dilution in MS liquid medium supplemented with 3% sucrose at 10-min intervals (*6*). Embryogenic callus is dipped in MS liquid medium containing 0.5 *M* sucrose for 3 h. The pre-treated callus was re-hydrated by half-dilution in MS liquid medium supplemented with 3% sucrose at 10-min intervals (*8*).

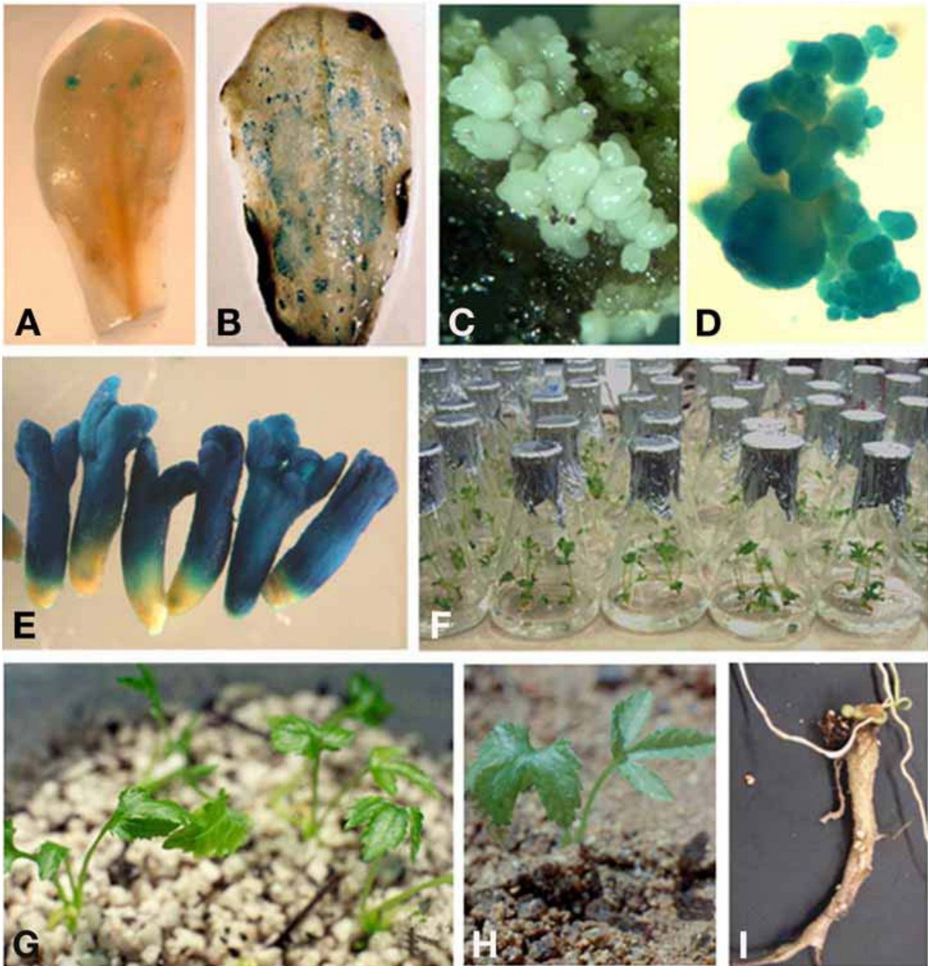

Fig. 1. Production of transgenic ginseng plants after co-cultivation of *A. tumefaciens* harboring pIG121 and cotyledon explants. (**A**) Transient expression of GUS gene in ginseng cotyledon without any treatment. (**B**) Transient expression of GUS gene in ginseng cotyledon pretreated with 0.5 *M* sucrose for 24 h. (**C**) Somatic embryo development from embryogenic callus on selection medium with 50 mg/L kanamycin. (**D**) GUS expression in somatic embryos. (**E**) GUS expression in cotyledonary somatic embryos. (**F**) Mass propagation of transgenic plantlets. (**G**) Transgenic plantlets acclimatized in autoclaved Perlite and peatmoss mixture. (**H**) Soil transferred transgenic ginseng plants. (**I**) Thickened roots of transgenic ginseng after natural senescence.

3.4. Cocultivation (48–72 h)

This step allows the bacteria in contact with the plant cells to transfer the T-DNA to the plant genome. During this period, the transformation process occurs.

1. Centrifuged bacterial solution re-suspended into EC liquid medium is suspended in the sterilized Petri dish. Cotyledon explants or embryogenic callus are dipped into bacterial liquid medium for about 10 min.
2. Under the sterile laminar flow, remove the bacterial solution with a pipet.
3. Culturing explants are blotted on the sterilized 90-mm filter paper in Petri dish for about 10 min to remove the remnant bacterial solution (*see* **Note 5**).
4. Transfer the culturing explants to EC medium without antibiotics for gene transfer into plant genome.
5. The plates are then sealed with Parafilm and incubated for a maximum of 2 or 3 d in the dark under culture room conditions (24°C). During this co-cultivation step, the agrobacteria should not over-grow on the explants.
6. Without addition of acetosyringone, transformation of *P. ginseng* is successfully achieved. However, treatment of acetosyringone (20–50 mg/L) enhances the frequency of transformation rate (data not shown).

3.5. Selection of Transformed Callus (10 to 15 wk)

This step will allow the growth of embryogenic callus and selection of transgenic calli on medium with 2,4-D and antibiotics. Selection of transgenic callus is the most important step for successful transformation.

1. The culturing explants (cotyledons or embryogenic callus) are removed from *Agrobacterium* co-cultivation medium after 2 to 3 d of culture (*see* **Note 10**).
2. The explants are transferred to new EC medium with 400 mg/L cefotaxime (to eliminate the bacteria). At this time, the medium do not contain the antibiotic for selection of transformed cells.
3. After allowing of callus growth for two weeks, explants are transferred to selection medium containing both 400 mg/L cefotaxime and 50 mg/L kanamycin (*see* **Note 6**) and subcultured at 3 wk intervals.
4. Plates are sealed with Parafilm and placed in the dark in the culture room (24°C) for 5 to 6 wk. Check the plates regularly to avoid contamination of bacteria.
5. The antibiotic resistant callus can be seen after three to five subcultures. The antibiotic resistant callus is more conspicuous when the subculture is repeated to about five times. Embryogenic callus is seen as white or opaque yellow on the senescenced callus.

3.6. Embryogenesis (3 to 6 wk)

Embryogenic callus (*see* **Fig. 1D**) is transferred to hormone-free somatic embryo induction (SI) medium to induce somatic embryo formation (*see* **Fig. C,D**). During the somatic embryo development, temporary omission of kanamycin is

effective for inducing somatic embryos (*see* **Note 11**). After induction of somatic embryos, they are transferred to selection medium.

1. Transfer the embryogenic calli to SI medium and place them in the light (40 µmol/m^2/s; in the in vitro growth chamber (24°C, 16/8-h light/dark photoperiod).
2. Calli are sub-cultured to new SI medium every 3 wk until the early somatic embryos appear.
3. Somatic embryos are formed from embryogenic callus between 30 and 50 d after the transfer to the SI medium (*see* **Fig. 1C–E**).

3.7. Plantlet Conversion and Growth (2 to 6 wk)

1. Cotyledonary somatic embryos do not germinate because of physiological dormancy (*see* **Note 12**).
2. To induce germination, cotyledonary somatic embryos are transferred onto embryo germination (EG) medium ($^1/_2$ MS medium with 5 mg/L GA_3 and 0.25% Gelrite). Germination occurs rapidly and converted into plantlets after 3 wk of culture.
3. When germinated plantlets are transferred to plantlet growth (PG) medium ($^1/_2$ MS medium lacking NH_4NO_3) in 100-mL Erlenmeyer flask (*see* **Fig. 1F**), root growth of plantlets is fostered (*see* **Note 13**).

3.8. Hardening and Soil Transfer of the Transgenic Plants (2 mo)

The plantlets transferred from the in vitro conditions to the greenhouse are very sensitive to fungus (e.g., *Botrytis*) (*8*). Thus, to avoid fungus attack, plants should be pre-cultured in an autoclavable large culture box containing Perlite and peatmoss mixture (3:1 [v/v]) which should be kept under sterilized conditions (*see* **Fig. 1G**). The culturing of plants in the sterilized culture matrix for about 2 mo results in the root thickening, at which time they are ready for exposure to normal greenhouse conditions.

1. Plantlets that have developed leaves and roots are transplanted into a polycarbonate or polyvinyl box containing sterilized Perlite and peatmoss mixture (3:1 [v/v]) covered with a transparent lid (*see* **Fig. 1G**) (*see* **Note 14**). The lid have pore (1.5-cm diameter) sealed with membrane filter (0.45 µm) for exchanging air. The whole plastic box containing moisture soil is sterilized for culturing of transgenic plants.
2. Under laminar flow, plants are transplanted in autoclaved soil and cultured in culture room for 2 mo. Ginseng plants are very sensitive to fungus attack, thus opening of lid should be done after about 2 mo of hardening.
3. If the culture room temperature is below 15°C, fungus attack after opening of lid is not severe because this temperature is unsuitable for fungus growth. Therefore, the low temperature incubator is good for soil hardening. In a low temperature incubator, the plant can be exposed to air conditioning. The plants are then watered alternately with water.
4. When the temperature regime of the uncontrolled greenhouse conditions are about 10 to 15°C and night conditions are about 4 to 10°C, acclimatized ginseng plants

can be transferred to pots with a mixture of soil: sand in the greenhouse (*see* **Fig. 1H**).

5. Complete soil transfer of transgenic plant will take 4 to 6 mo. Approximately 50% of the transferred plantlets will survive the greenhouse transfer and develop into plants. The plants have thickened roots after defoliation of shoots by natural senescence (*see* **Fig. 1I**).
6. Although ginseng plants are successfully transferred to soil, they will only produce flowers after 3 to 4 yr. Therefore, loss of plants is a serious problem before seed set.

4. Notes

1. *P. ginseng* C.A. Meyer seeds for transformation experiments can be requested from Dr. YE Choi (*yechoi@kangwon.ac.kr*).
2. Various laboratory-disarmed strains can be used. *Agrobacterium* strain LBA4404 and GV3101 are efficient for transformation of *P. ginseng*.
3. To induce embryogenic callus, cotyledon explants are cultured on full-strength MS ***(10)*** solid medium with 1.0 mg/L 2,4-D. Hormone-free agar medium is used to induce somatic embryos from embryogenic callus. Germination of somatic embryo requires 5 mg/L GA_3 treatment for 2 wk. To stimulate the balanced root growth of plantlets, modified MS medium lacking NH_4NO_3 is necessary ***(11)***.
4. We have used two kinds of gelling agent. Agar is better than Gelrite not only for induction of embryogenic callus but also for inducing somatic embryos from embryogenic callus. For germination and further growth of plants, Gelrite is more effective.
5. Blotting of explants on filter paper to remove the remnant bacteria after dipping into bacterial solution is not only effective to prevent *Agrobacteria* overgrowth but also to enhance the transformation frequency.
6. The size of embryogenic callus and inoculation number of explants on the Petri dish are important factors for selective growth of transformed callus. Embryogenic calli should be separated in small clumps less than 5 mm in size. About 20 embryogenic calli are cultured per Petri dish. The bigger the size and larger the number of callus inoculation, the higher the concentration of kanamycin that is required.
7. Cotyledon explants of zygotic embryos are the best explants to induce somatic embryos and/or embryogenic callus induction. *P. ginseng* seeds contain rudimentary zygotic embryos after harvest. Therefore, moist-chilling treatment of seeds for several months is required to support the growth and maturation of zygotic embryos ***(13)***.
8. Embryogenic callus is preferable to induce high frequency transformation. However, cotyledon explants can be used as culturing explants. In this case, embryogenic callus should be selected from transgenic calli after repeated subculture on the selection medium. In *P. ginseng*, once embryogenic callus was induced, they were easily maintained by subculturing onto MS medium with 1.0 mg/L 2,4-D and 3.0 mg/L sucrose solidified with 0.9% agar. Specific photoperiods and light illumination are not needed.

9. Cotyledon explants or embryogenic callus can be used for genetic transformation. However, plasmolyzing pretreatment greatly enhances the transformation frequency (*6,8*). Cotyledons of zygotic embryos are strongly resistant to the plasmolyzing solution (1.0 *M* sucrose). However, embryogenic callus is very sensitive to plasmolyzing solution and requires treatment for short periods of time (not more than 30 min) with 1.0 *M* sucrose.
10. If *Agrobacterium* is overgrown on the medium, the explants are transferred into EC liquid medium containing 500 mg/L cefotaxime and shake-cultured for about 2 h. Thereafter, the explants are blotted on sterilized filter paper.
11. In *P. ginseng*, selection by kanamycin is not a problem for selection of transformants. The appropriate concentration of kanamycin is about 25 to 100 mg/L. The range of kanamycin concentration should be varied by the size of callus. Appropriate hygromycin concentration is about 10 to 30 mg/L. Hygromycin treatment is very effective to select the transgenic callus and embryos, but growth of transgenic callus can be suppressed by the influence of browning of damaged callus.
12. Ginseng somatic embryos have a tendency for physiological dormancy similar to zygotic embryos (*15*). GA_3 (5 mg/L) treatment effectively broke the dormancy of somatic embryos. Without GA_3 treatment, somatic embryos do not germinate and their is no shoot growth after root germination.
13. On full strength MS medium, root growth of plantlets does not do well. Lowering the medium strength or omitting of NH_4NO_3 enhances the root growth of plantlets (*11*).
14. Before transfering plants to the greenhouse they should be kept in a sterilized culture chamber containing soil because ginseng plants are very sensitive to air-inborn fungus such as Botrytis. Before culture in sealed box, the soil should be watered to maintain humidity. Culture boxs have air pores protected by membrane filters.

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Hemp (*Cannabis sativa* L.)

Mistianne Feeney and Zamir K. Punja

Summary

Hemp (*Cannabis sativa* L.) suspension culture cells were transformed with *Agrobacterium tumefaciens* strain EHA101 carrying the binary plasmid pNOV3635. The plasmid contains a phosphomannose isomerase (PMI) selectable marker gene. Cells transformed with PMI are capable of metabolizing the selective agent, mannose, whereas cells not expressing the gene are incapable of using the carbon source and will stop growing. Callus masses proliferating on selection were screened for PMI expression using a chlorophenol red assay. Genomic DNA was extracted from putatively transformed callus lines and the presence of the PMI gene was confirmed using polymerase chain reaction and Southern hybridization. Using this method, an average transformation frequency of 31.23% ± 0.14 was obtained for all transformation experiments, with a range of 15.1 to 55.3%.

Key Words: Hemp; *Cannabis sativa*; *Agrobacterium tumefaciens*; *Agrobacterium*-mediated transformation; phosphomannose isomerase; mannose selection; chlorophenol red assay; transformation protocol; callus; suspension culture; plant tissue culture.

1. Introduction

Hemp (*Cannabis sativa* L.) is regaining importance as a cultivated crop after decades of legal prohibitions. Hemp cultivation is now permitted in Canada under strict governmental control. The regulatory concern arises because the species, *Cannabis sativa*, has been selected for very different qualities; hemp varieties are cultivated for seed, oil, and fiber and may contain only trace amounts of the drug Δ^9-tetrahydrocannabinol (THC), whereas marijuana varieties are bred for high THC content *(1)*. Morphologically, hemp and marijuana varieties are difficult to tell apart.

There is interest in developing improved varieties of hemp that are resistant to disease and pest pressures and possess enhanced qualities *(1,2)*. Transformation

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technology can make available a greater amount of genetic diversity towards hemp improvement. Only a small number of hemp tissue culture and transformation reports have been published. At present, there is no established protocol for regeneration of hemp, either from organogenesis or somatic embryogenesis. The current consensus is that callus production from hemp explants is relatively straightforward. Callus readily produces roots, but has a very poor ability for shoot formation *(2–5)*. In the only report of hemp transformation of which we are aware, MacKinnon et al. *(2)* avoided the need for a regeneration protocol by using an alternative *Agrobacterium*-mediated transformation procedure to introduce a gene into shoot tips. Our objective was to demonstrate that gene transfer can occur in callus cultures, with the anticipation that a regeneration protocol will be established involving an intervening callus phase.

This chapter describes the *Agrobacterium*-mediated transformation of hemp callus with the selectable marker gene phosphomannose isomerase (PMI). The PMI gene confers a metabolic advantage to the plant cell, allowing growth on a selective medium containing a sugar, mannose, as the selective agent. Methods are outlined for the initiation and establishment of hemp callus and suspension cultures. Callus growing on selection are screened for PMI expression using a biochemical assay. DNA is extracted from putatively transformed callus lines and analysed by polymerase chain reaction (PCR) and Southern hybridization techniques to detect the gene of interest. An average transformation frequency of 31.23% ± 0.14 was obtained for all transformation experiments, with a range of 15.1 to 55.3%. This value represents an average of 31 mannose-metabolizing independent events in 100 explants targeted for transformation.

2. Materials

2.1. Hemp Tissue Culture

1. Hemp seeds cv. Anka are monoecious and cultivated for seed.
2. Potting mix soil: Sunshine Mix no. 1 (Sun Gro Horticulture, Bellevue, WA).
3. Commercial bleach: Javex containing 4.5% NaOCl. For sterilization, dilute with double distilled water (ddH_2O) to 10% (v/v) Javex.
4. Tween-20 (polyethylene sorbitan monolaurate) surfactant (Bio Rad, Hercules, CA).
5. Filter paper: Whatman no.1, 70 mm-diameter filter paper (Whatman Int. Ltd., Cambridge, UK).
6. Plant growth regulator (PGR): 1000 μ*M* stock solutions. Dissolve PGRs in a small amount of 1 *N* NaOH (for kinetin or 6-furfurylaminopurine [kinetin]; Sigma, St. Louis, MO) or 70% ethanol (for 2,4-dichlorophenoxyacetic acid [2,4-D]; Sigma, St. Louis, MO) and bring to volume with ddH_2O. Store at 4°C. Kinetin and 2,4-D can be co-autoclaved with media.
7. MB5D1K: Murashige and Skoog (MS) macro- and micro-nutrients *(6)* (1900 mg/L KNO_3, 1650 mg/L NH_4NO_3, 180 mg/L $MgSO_4$, 170 mg/L KH_2PO_4, 16.9 mg/L $MnSO_4{\cdot}H_2O$, 6.2 mg/L H_3BO_3, 8.6 mg/L $ZnSO_4{\cdot}7H_2O$, 0.83 mg/L KI,

0.025 mg/L $CuSO_4 \cdot 5H_2O$, 0.25 mg/L $Na_2MoO_4 \cdot 2H_2O$, 0.025 mg/L $CoCl_2 \cdot 6H_2O$, 440 mg/L $CaCl_2 \cdot 2H_2O$, 27.8 mg/L $FeSO_4 \cdot 7H_2O$, and 37.3 mg/L Na_2-EDTA), Gamborg B5 vitamins *(7)* (1 mg/L nicotinic acid, 1 mg/L pyridoxine-HCl, and 10 mg/L thiamine-HCl), 0.1 g/L *myo*-inositol, 30 g/L sucrose, 8 g/L bacteriological agar (Anachemia Canada Inc., Montreal, PQ), 5 μ*M* 2,4-D, and 1 μ*M* kinetin, pH 5.8.

8. MB2.5D: MS macro- and micro-nutrients, Gamborg B5 vitamins, 0.1 g/L *myo*-inositol, 30 g/L sucrose, 8 g/L bacteriological agar (for solid medium), 2.5 μ*M* 2,4-D, pH 5.8.
9. Parafilm (Pechiney Plastic Packaging, Chicago, IL).

2.2. Agrobacterium *Culture Conditions*

1. *Agrobacterium tumefaciens* strain EHA101 *(8)* contains plasmid pNOV3635 as a binary vector *(5)*. The plasmid pNOV3635 carries a PMI gene under control of the *Arabidopsis thaliana* ubiquitin promoter (*Ubq3*) and the nopaline synthase terminator (NOS). Spectinomycin and kanamycin selectable markers are present on the pNOV3635 plasmid and the Ti-plasmid carrying the virulence genes, respectively.
2. LB (Luria-Bertani medium): 10 g/L bacto-tryptone, 5 g/L bacto-yeast extract, 10 g/L NaCl, and 15 g/L bacteriological agar (for solid medium).
3. Spectinomycin dihydrochloride: Dissolve in ddH_2O at 0.25 *M*. Filter-sterilize using a 0.2 μm filter and store in aliquots at –20°C (Sigma, St. Louis, MO).
4. Kanamycin monosulfate: Dissolve in ddH_2O at 0.17 *M*. Filter-sterilize using a 0.2 μm filter and store in aliquots at –20°C (Sigma, St. Louis, MO).
5. *Agrobacterium* cultures are centrifuged using a Beckman GS-6R Centrifuge (Beckman Coulter Inc., Fullerton, CA).
6. Acetosyringone (3,5-Dimethoxy-4′-hydroxyacetophenone): Dissolve in a small quantity of 100% methanol. Dilute with ddH_2O at 100 m*M*. Filter-sterilize using a 0.2 μm filter and store in aliquots at –20°C (Sigma-Aldrich, Milwaukee, WI).

2.3. Transformation

2.3.1. Inoculation and Co-cultivation

1. Filtration funnels: A Coors porcelain Hirsch funnel with a fixed perforated plate or a Coors porcelain Buchner funnel with a fixed perforated plate can be used (Fischer Scientific, Pittsburgh, PA).
2. Filter paper: Whatman no. 1, sized to fit the perforated funnel plate (Whatman Int. Ltd., Cambridge, UK).

2.3.2. Selection

1. Timentin: Dissolve in double distilled water (ddH_2O) at 300 mg/mL. Filter-sterilize using a 0.2 μm filter. Prepare fresh before each use (SmithKline Beecham, Oakville, ON).
2. Mannose (D-mannopyranose): Dissolve in media with other components (Sigma).

2.4. PMI Assay

1. Chlorophenol red: Dissolve in a small amount of 70% ethanol and add to assay medium. Chlorophenol red has a strong, unpleasant smell and should be dispensed while wearing gloves in a fumehood (Sigma-Aldrich, Milwaukee, WI).
2. PMI assay media: MB2.5D supplemented with either 20 g/L mannose (selection) or 30 g/L sucrose (control), 0.1 g/L chlorophenol red, and 8 g/L bacteriological agar, pH 6 (*see* **Note 1**).
3. Enzyme-linked immunosorbent assay (ELISA) plates (Becton Dickinson and Co., Lincoln Park, NJ).

2.5. Genomic DNA Extraction and Molecular Analysis

2.5.1. Genomic DNA Extraction

1. Grind callus samples using a plastic pellet pestle (Kontes Glass Company, Vineland, NJ) attached to a hand-held drill.
2. PVPP (polyvinylpolypyrrolidone) (Sigma, St. Louis, MO).
3. Silica sand: Approximately 50 g of sand in a glass jar autoclaved at 121°C and 15 to 20 psi for 25 min (Sigma, St. Louis, MO).
4. DNeasy® AP1 buffer: A component of the Qiagen DNeasy® Plant Mini Kit (Qiagen, Valencia, CA).
5. 100 mg/mL RNase A: A component of the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA).
6. Qiagen DNeasy® Plant Mini Kit (Qiagen, Valencia, CA).

2.5.2. PCR

1. Primers: consist of two 18-nucleotide sequences (40 n*M* each) PMI-1 5′-ACAGC-CACTCTCCATTCA-3′ and PMI-2 5′-GTTTGCCATCACTTCCAG-3′ *(**9**)*. Dilute to 1.5 μ*M* with sterile distilled water. Store at –20°C.
2. 10X PCR buffer: 200 m*M* Tris-HCl and 500 m*M* KCl. Store at –20°C (Invitrogen, Burlington, ON).
3. 50 m*M* $MgCl_2$: Store at –20°C (Invitrogen, Burlington, ON).
4. Ultrapure dNTP set: Stock solutions are made at 10 m*M* each dATP, dTTP, dGTP, dCTP. Store at –20°C (Amersham Biosciences, Piscataway, NJ).
5. Taq polymerase: Store at –20°C (Invitrogen, Burlington, ON).
6. Amplification is carried out using a DNA Thermal Cycler 9700 (PE Applied Biosystems, Mississauga, ON).

2.5.3. Southern Hybridization

1. *Hin*dIII: store enzyme at –20°C (Gibco BRL Life Technologies, Burlington ON).
2. Nylon membrane: Positively charged Hybond-XL membrane (Amersham Biosciences, Piscataway, NJ).
3. Radiolabeled probe: PCR amplification of the 550 bp PMI gene fragment, substituting a P^{32}-labeled dCTP. The PCR product is purified using a QIAquick PCR Purification Kit (Qiagen, Mississauga, ON).
4. X-ray film: Kodak X-OMAT.

3. Methods

3.1. Hemp Tissue Culture

1. Sow hemp seeds in 5-cm^2 plastic containers containing moistened potting mix soil at ambient room temperatures (21–24°C). Place seedlings under cool-white fluorescent lights with an intensity of 18 µmol/m^2/s and a 12-h photoperiod (*see* **Note 2**).
2. By 4 wk, seedlings grow to a height of about 20 cm and have 2 to 4 pairs of true leaves. Cut seedlings at the base of the stem, approx 1 to 1.5-cm from the soil.
3. Surface sterilize the seedlings by immersion in 70% ethanol for 20 s, followed by 10% commercial bleach containing 2 drops of 0.1% Tween-20/100 mL for 1 min, while stirring gently. Rinse three times with sterile distilled water.
4. Transfer seedlings to sterile Petri dishes lined with moistened filter paper. Excise stem (0.5-cm long) and leaf (0.5 cm^2) sections and transfer explants to MB5D1K solid medium. Wrap dishes in Parafilm and place cultures in the dark at ambient room temperature for 1 mo for callus development (*see* **Note 3**).
5. To initiate suspension cultures, callus developing on explants are cut into small pieces. Transfer 0.5 to 1 mg of callus to 20 mL of MB2.5D liquid medium in 150-mL Erlenmeyer flasks. Cap flasks with a double layer of aluminum foil and shake at 115 rpm at ambient room temperature with 12-h/d light at an intensity of 10 µmol/m^2/s.
6. Subculture suspension cultures at 2-wk intervals by discarding $^3/_4$ of the spent medium and replacing with fresh medium.
7. At 4 wk, transfer suspensions to 50 mL of MB2.5D liquid medium in 250-mL Erlenmeyer flasks by suctioning 1 mL packed cell volume through a 3-mm diameter pipet tip. Shake cultures at 150 rpm.

3.2. Agrobacterium Culture Conditions

1. *Agrobacterium tumefaciens* strain EHA101 containing the binary vector pNOV3635 is used for hemp transformation. Inoculate 25 mL of LB liquid medium with 2 colonies of *Agrobacterium*. To retain the Ti-plasmid and pNOV3635 within the bacteria, LB is supplemented with 50 mg/L of kanamycin and 150 mg/L of spectinomycin, respectively. Shake culture at 200 rpm at 28°C for 48 h (*see* **Note 4**).
2. Collect bacterial cells by centrifugation at 3700*g* for 20 min.
3. Wash pellet with MB2.5D and resuspend in MB2.5D containing 100 µ*M* of acetosyringone to a final OD_{600nm} 1.6 to 1.8. Incubate culture for 10 min with occasional stirring in the laminar flow hood prior to inoculating plant cells.

3.3. Transformation

3.3.1. Inoculation and Co-cultivation

1. Using a 3-mm wide-mouth pipet, transfer 1 mL (packed cell volume) of hemp suspension cell clumps along with 4 mL of MB2.5D to a sterile Petri dish.
2. Inoculate suspension cells with 5 mL of *Agrobacterium* culture for 30 min with occasional stirring.

3. Meanwhile, a vacuum filtration apparatus is assembled in the flow hood. Place a clean support stand in the flow hood. Fasten a 2-L sterile glass filtering flask to the support using a flask clamp. Attach tubing from the glass filtering flask to a vacuum source. A clean rubber stopper with a hole is fitted over the mouth of the filtering flask into which a sterile funnel is placed. The filtering assembly requires a tight fit to make a seal and produce a vacuum.
4. Collect hemp cell clumps onto a filter paper by vacuum filtration.
5. Transfer the filter paper and suspension cells to MB2.5D solid medium. Wrap dishes with Parafilm and co-cultivate for 3 d in the dark at ambient room temperature.

3.3.2. Selection

1. Gently scrape suspension cells onto a fresh moistened filter paper placed in a sterile filtering funnel which is fitted onto the vacuum filtration apparatus. Rinse 3 times with a total volume of 200 mL of MB2.5D to wash off bacteria.
2. To further eliminate *Agrobacterium* after washing, transfer the filter supporting cell clumps to MB2.5D solid medium containing 300 mg/L of timentin. Wrap dishes with Parafilm and place in the dark for 7 d.
3. Transfer small individual callus clumps to MB2.5D solid medium containing 1% (w/v) mannose and 300 mg/L of timentin (*see* **Note 5**). Wrap dishes with Parafilm and place in the dark for 4 wk (*see* **Note 6**). The appearance of callus after 4 wk of incubation is shown in **Fig. 1 A–C**.
4. Transfer growing callus to MB2.5D containing 2% (w/v) mannose and 150 mg/L of timentin for 4 wk in the dark.

3.4. PMI Assay

Most plant cells and tissue cultures are dependant on a carbon source, often sucrose, supplemented in the medium. Mannose is recognized as a carbon source that cannot support growth of most explants because of their inability to metabolize the sugar ***(10)***. The PMI selection strategy makes use of mannose as a selection agent. Cells that express the PMI gene are conferred a metabolic advantage over cells lacking the gene. Growth of transgenic tissue is supported on mannose-containing media, while nontransformed tissue either stops growing or dies from starvation ***(10,11)***.

The chlorophenol red assay ***(12)*** is a quick and easy method to screen for the expression of the PMI gene in putatively transformed cells. The assay is based on the observation that actively growing plant cells acidify their surroundings ***(12)***. Chlorophenol red is an indicator dye that is sensitive to pH changes. It can be incorporated into the mannose selection medium and will produce a color change as the pH of the medium decreases ***(11)***. A color change from red to yellow in the assay medium reflects callus growth, indicating that the sample expresses the PMI gene. Callus unable to actively grow and metabolize in the presence of mannose do not acidify the medium and the

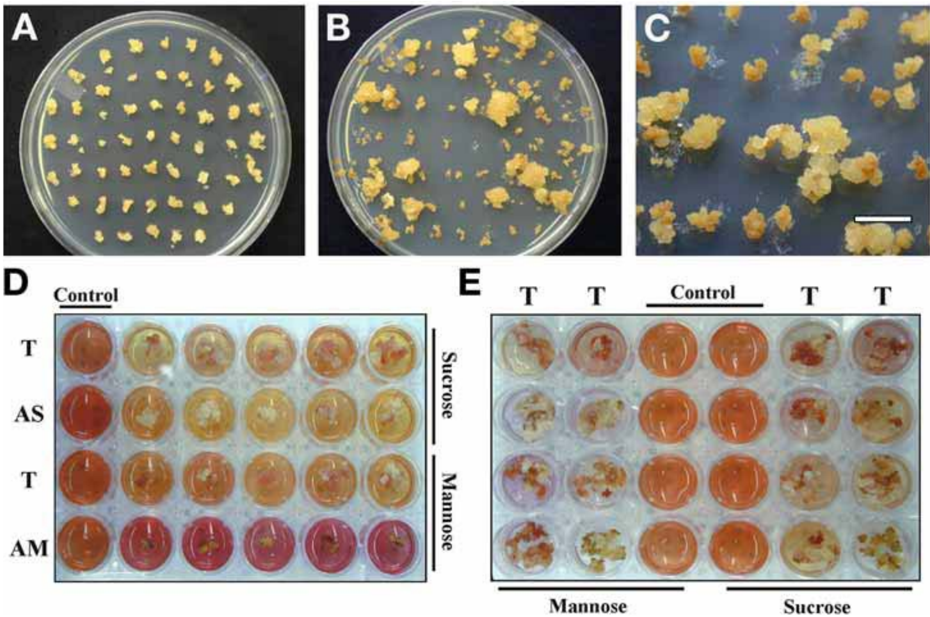

Fig. 1. Selection of Anka callus transformed with pNOV3635 on MB2.5D with 300 mg/L timentin and 1% mannose after 4 wk. **(A)** Nontransformed cell masses are arrested in growth. **(B)** Transformed cell masses are distinguished by their enlarged size compared to untransformed callus. **(C)** Transformed callus on mannose medium, forming large, pale yellow callus protruding from small, dark-yellow parental callus. Photos are of 9-cm diameter dishes. Scale bar: 5 mm for (C). **(D,E)** Chlorophenol-red PMI assay after 3 to 4 d. Medium is composed of MB2.5D, 8 g/L agar, and 0.1 g/L chlorophenol red with either 3% sucrose or 2% mannose. Control wells do not contain callus. (*T*) Transformed callus harboring the PMI gene grew on both sugar sources, turning the medium pale yellow. (*AS*) Nontransformed callus metabolized sucrose and acidified the medium, turning it a pale yellow color. (*AM*) Callus incubated with *Agrobacterium* lacking the PMI plasmid did not acidify the medium. Well diameter in each dish is 1.5 cm. (Reproduced with permission from **ref. 5**).

color remains red. All callus lines testing positive for PMI expression in the chlorophenol red assay were confirmed to carry the PMI gene in PCR assays (*see* **Note 7**).

1. PMI assay media are dispensed in 600-µL aliquots into each well of a sterile 24-well ELISA plate (*see* **Note 8**).
2. Transfer callus pieces (approx 0.6 cm^2) into each well of the ELISA plate. Wrap plate with Parafilm and incubate in the dark for 3 d.
3. Record color changes in each well and photograph the ELISA plate (*see* **Note 9**). Sample PMI assays are depicted in **Fig. 1 D,E**.

3.5. Genomic DNA Extraction and Molecular Analysis

3.5.1. Genomic DNA Extraction

1. DNA is extracted from callus following a modified protocol (*13*). Grind each 100 mg callus sample with 25 mg of PVPP, 100 mg of sterile silica sand, 200 µL of DNeasy AP1 buffer, and 4 µL of RNase A in a 1.5-mL microfuge tube.
2. Add an additional 200 µL of AP1 buffer to each sample, vortex, and isolate DNA following the Qiagen DNeasy kit procedure.
3. Genomic DNA is stored at –20°C.

3.5.2. PCR

1. Assemble all reagents for the PCR reaction. Each 25 µL PCR reaction contains 1.5 m*M* of $MgCl_2$, 20 m*M* of Tris-HCl, 50 m*M* of KCl, 200 µ*M* of each dNTP, 0.2 µ*M* of each primer, 2 units of Taq polymerase, 5 µL of template DNA (of appropriate dilution), and sterile distilled water to volume.
2. Primers amplify a 550-bp region within the PMI gene (*9*). PCR conditions: initial denaturation step of 3 min at 95°C followed by 30 cycles of 30 s at 95°C (template denaturation), 30 s at 55°C (primer annealing), and 45 s at 72°C (DNA synthesis), with a terminal elongation step of 5 min at 72°C.
3. Once the reaction is complete, PCR products can be stored at 4°C or –20°C until further analysis, or samples can be run on a 0.9% agarose gel and visualized by illumination with ultra-violet light.

3.5.3. Southern Hybridization

1. Digest genomic DNA with *Hin*dIII at 37°C overnight.
2. Electrophorese samples on a 0.8% agarose gel.
3. Transfer DNA fragments from the gel to a nylon membrane by capillary transfer with 0.4 *M* NaOH using a downward blot assembly (*14*).
4. Hybridization of the ^{32}P-labeled probe to the filter is performed according to the Amersham protocol for Hybond-XL membranes.
5. Expose membrane to X-ray film in the presence of an intensifying screen for 3 to 24 h at –80°C.

4. Notes

1. Assaying other hemp varieties or tissue types may require adjusting the concentration of chlorophenol red within the assay medium. Higher concentrations of chlorophenol red (up to 2 mg/mL) incorporated into the PMI assay medium caused transgenic callus to absorb the dye and produced no color change within wells. The callus did not resume growth when transferred back to mannose or sucrose-containing medium. An excess uptake of chlorophenol red from the assay medium may arrest tissue metabolism and cause cell death.
2. Hemp seedlings attract thrip insect pests. Thrip eggs are difficult to see and can survive the sterilization process and ruin experiments. A thrip predatory mite (*Amblyseius cucumeris*) can be successfully used as biological control to significantly

lower or eliminate the thrip population before a new batch of seeds are planted for experiments. The predatory mites have not interfered with experiments and can be purchased from local garden stores.

3. After sterilization, it is important to place seedling material onto sterile moistened filter paper while cutting explants. Seedling tissues quickly wilt under flow hood conditions, which may affect callus development. The moistened filters keep tissues turgid during processing.
4. Long-term *Agrobacterium* cultures should be stored at –80°C. To do this, grow an overnight culture and mix with 15% (v/v) sterile glycerol. Vortex and transfer 1 mL to a cryogenic tube, label, freeze in liquid nitrogen, and store at –80°C. To start a culture for experiments, scrape cells from the frozen culture with a sterile loop and streak an LB plate containing the appropriate selection.
5. Hemp suspension cells are moved to different treatments on a filter paper support for ease of transfer. Individual cell clumps (without filter paper support) are transferred to selection to maintain better contact with the medium.
6. Callus turns from pale yellow to a darker yellow color within 1 wk of being placed on 1% mannose selection medium. By 4 wk, cells capable of metabolizing mannose are easily distinguished by their color and larger size. Pale yellow callus emerges from darker yellow cell clumps, growing larger than other callus masses.
7. We found two callus lines that were not positive for gene expression in the PMI assay but were confirmed to harbor the PMI gene by PCR analysis. It is possible that the PMI assay may not be sensitive enough to detect low expressing transgenic tissue, or that the PMI gene was silenced.
8. Over time, the color of the PMI assay medium can fade from red to a pale red/orange color and the small quantity of assay medium within wells dries out quickly from water evaporation, so it is recommended to use freshly prepared assay medium.
9. Control callus not containing the PMI gene (incubated with EHA101 lacking pNOV3635) can give false-positive results when assayed for PMI activity. When transferred to mannose-containing assay medium, control callus will produce a color change, suggesting that it can metabolize mannose. However, subculture to fresh assay medium produces no color change, indicating that callus cannot survive on mannose-containing media. Control callus may store sugar reserves when grown on sucrose-containing medium. Upon transfer to mannose-containing medium, the sugar reserves are utilized for metabolism, acidifying the assay medium. To eliminate false-positive results, the control callus is incubated for 1 wk on mannose-containing medium prior to analysis by the PMI assay.

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analyses for THC content over the duration of the study to ensure they did not exceed the legal allowable limit of 0.3% THC. The cultures were grown under permit no. 00-F0041-R-01 and disposed of according to the requirements.

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Opium Poppy (*Papaver somniferum*)

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Summary

The genetic transformation of opium poppy, *Papaver somniferum,* offers the opportunity to study the mechanisms involved in the regulation of benzylisoquinoline and morphinan alkaloid biosynthesis. The development of an efficient transformation protocol for opium poppy has allowed us to transform a range of genotypes from all around the world, including previously recalcitrant high-yielding commercial Australian cultivars. The method involves *Agrobacterium tumefaciens* infection of hypocotyl explants, followed by the production of antibiotic or herbicide resistant embryogenic callus, the subsequent induction of somatic embryos and development into normal plants. The use of different selective agents, binary vectors, and poppy genotypes has demonstrated the robustness and reliability of this protocol in the production of many hundreds of confirmed transgenic poppies.

Key Words: *Agrobacterium tumefacien*; opium poppy; *Papaver somniferum*; somatic embryogenesis; transformation.

1. Introduction

The process of somatic embryogenesis is well known and well described for a wide variety of plant species in vitro. The regeneration of plants from cultured *Papaver somniferum* tissues has been reported by various authors ***(1–6)*** mainly via somatic embryogenesis. However, poppy explants and cultures have proved particularly vulnerable to problematic levels of tissue browning, low frequencies of embryo or shoot formation, and poor success rates in transferring rooted plants to soil.

These problems have been largely overcome by a number of protocol modifications and we were able to develop an efficient *Agrobacterium tumefaciens* mediated transformation system based on somatic embryogenesis. The most influential modifications involved enhanced pH buffering of a standard tissue

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culture medium, and the bottom cooling of culture dishes to achieve more normal plantlet formation *(7)*.

Production of transgenic poppy plants includes the following procedures: (1) germination of mature sterilized seed, growth of seedlings and preparation of hypocotyl explants; (2) *Agrobacterium* infection of hypocotyl explants and co-cultivation; (3) production of embryogenic callus cultures and growth of somatic embryos; (4) development of somatic embryos to normal plantlets; and (5) transfer of transgenic plants to soil.

We define transformation efficiency in our system as the percentage of hypocotyl explants producing somatic embryos that develop into transgenic plantlets able to be transplanted to soil. The highest transformation efficiency achieved was 48% in an Iranian genotype, and the highest efficiency in an Australian commercial cultivar was 11%. Across many varied transformation experiments in Australian germplasm we have achieved an average of about 5% transformation efficiency. We have found this to be a workable and practical level for metabolic engineering research in this important pharmaceutical crop.

2. Materials

2.1. Plant Material

1. Mature seeds of opium poppy of commercial cultivars (Tasmanian Alkaloids, Westbury, Tasmania).
2. 5- to 7-d-old poppy seedlings.

2.2. Tissue Culture

2.2.1. Stock Solutions

1. 10X Gamborg's B5 Basal macronutrient *(8)* stock: 1.5 g/L of $NaH_2PO_4 \cdot 2H_2O$, 25 g/L of KNO_3, 1.34 g/L of $(NH_4)_2SO_4$, 2.5 g/L of $MgSO_4 \cdot 7H_2O$ and 1.5 g/L of $CaCl_2 \cdot 2H_2O$. Store at 4°C.
2. 1000X Gamborg's B5 Basal micronutrient (*8*) stock: 10 g/L of $MnSO_4 \cdot 4H_2O$, 250mg/L of $Na_2MoO_4 \cdot 2H_2O$, 3 g/L of H_3BO_3, 2 g/L of $ZnSO_4 \cdot 7H_2O$, 25 mg/L of $CuSO_4 \cdot 5H_2O$, 25 mg/L of $CoCl_2 \cdot 6H_2O$, 750 mg/L of KI. Make 100 mL of stock solution at a time and store at 4°C.
3. 100X Gamborg's B5 vitamin *(8)* stock: 100 mg/L nicotinic acid, 100 mg/L of pyridoxine HCl, 1 g/L of thiamine HCl, and 10 g/L of *myo*-inositol. Make 500 mL of stock solution at a time and store at 4°C.
4. 100X Iron stock: 2.8 g/L of Sequestrene 330 Fe (Fe diethylenetriamine pentaacetate). Make 500 mL of stock solution at a time and store at 4°C in brown glass.
5. 2,4-Dichlorophenoxyacetic acid (2,4-D): 1 mg/mL stock solution is prepared by dissolving 100 mg of powder in 1 mL of absolute ethanol, add 3 mL of 1 *N* KOH and adjust the volume to 80 mL with distilled water. pH is adjusted to 6.0 with 1 *N* HCl and the final volume is adjusted to 100 mL. Store at 4°C.

2.2.2. Media

1. B50: 1X B5 macronutrient stock, 1X B5 micronutrient stock, 1X B5 vitamin stock, 1X iron stock, 20 g/L sucrose, 2 g/L MES buffer, pH 5.6. Add 8 g/L agar (Sigma; cat.no. A1296) after pH adjustment and prior to autoclaving.
2. Callus Medium (CM): 1X B50 with 1 mL 2,4-D (1 mg/mL stock solution) added prior to pH adjustment and autoclaving.
3. *Agrobacterium* growth medium (MGL): 5 g/L of mannitol, 0.25 g/L of KH_2PO_4, 0.1 g/L of $MgSO_4{\cdot}7H_2O$, 2.5 g/L of yeast extract, 1 g/L of L-glutamic acid, 0.1 g/L of NaCl, 5 g/L of tryptone, 10 µL of biotin (0.1 mg/mL stock), pH 7.0. Autoclave in 100 mL volumes.

2.3. Other Chemicals

1. 25 mg/mL Paromomycin sulfate stock: Prepare by dissolving powder in sterile water, aliquot, and store at –20°C.
2. 10 mg/mL Phosphinothricin stock: Prepare by dissolving powder in water and sterilise by filtration, aliquot, and store at –20°C.
3. 20 mg/mL Rifampicin stock: Prepare by dissolving powder in methanol, aliquot, and store at –20°C.
4. 50 mg/mL Streptomycin stock: Prepare by dissolving powder in water and sterilize by filtration.
5. 50 mg/mL Spectinomycin stock: Prepare by dissolving powder in water and sterilize by filtration.
6. 150 mg/mL Timentin stock: Prepare by dissolving powder in sterile water or add powder direct to medium.
7. All the chemicals used for media are added to autoclaved medium once it has cooled to about 60°C. Swirl to mix thoroughly through the medium before pouring into 90 × 25-mm Petri dishes.

2.4. Culture Conditions

Seeds are germinated, hypocotyl explant material and rooted shoots are all maintained in a 24°C growth room with a 16-h photoperiod and lighting of approx 150 µmol/m^2/s. Embryogenic callus cultures are also maintained under these light conditions but sit on modified shelves through which cooled water circulates. The water temperature is maintained at 18 to 20°C.

2.5. Bacterial Strains and Binary Vectors

1. *Agrobacterium* strains: AGL0 and AGL1 ***(9)***.
2. Binary vectors: pBSF16 and COR1.1/2 hpRNA ***(7,10)*** (*see* **Fig. 1** for a brief description of the key components of these binary vectors).

3. Methods

3.1. Seed Germination and Explant Initiation

1. Mature seeds of *P. somniferum* are surface sterilized in 1.5-mL Eppendorf tubes by washing for 30 to 60s in 70% ethanol then in 1%(w/v) sodium hypochlorite

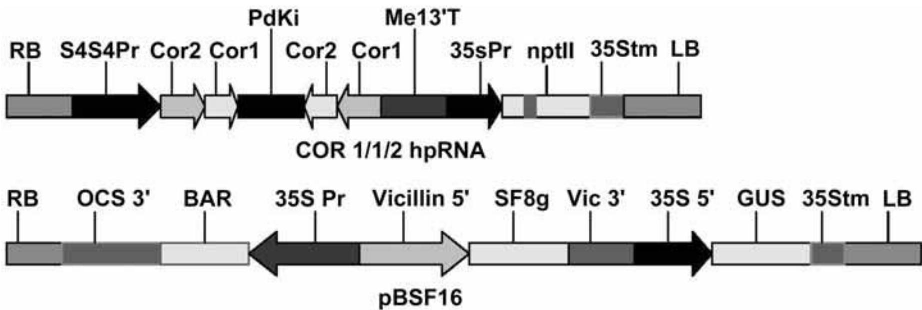

Fig. 1. T-DNA composition of two illustrative plasmids (COR 1.1/2 hpRNA and pBSF16) used in poppy transformation. LB and RB, the left and right borders of the T-DNA; S4S4Pr, subterannean clover stunt virus promoter 4; Cor1 and Cor2, codeinone reductase cDNA fragments; Pd Ki, ortho-phosphate dikinase intron; Me1 3′T, malic enzyme terminator; 35sPr, 35S promoter, nptII, *npt*II gene; 35Stm, 35S terminator; OCS 3′, octopine synthase terminator; BAR, *bar* gene; Vicillin 5, vicillin promoter; SF8g, sunflower seed albumin cDNA; Vic 3′, vicillin terminator; 35S 5′, 35S promoter; GUS, *gus* gene; 35Stm, 35S terminator.

solution plus 1 to 2 drops of autoclaved Tween-20 for 20 min with agitation (*see* **Note 1**).

2. Seeds are then tipped into 55 × 15-mm Petri dishes and rinsed 3 to 4 times in sterile distilled water or until no smell of bleach remains.
3. Rinsed seeds are placed on Petri dishes containing B50 agar medium with approx 30 to 50 seed/dish. Dishes are sealed with Parafilm and imbibed at 4°C for 24 to 48 h. Seeds are germinated at 24°C in a 16-h photoperiod.
4. Germination should be obvious 1- to 2-d later and seedlings should be approx 5- to 9-mm in length about 7 d after seeds are placed at 24°C.
5. Cotyledons should be obvious and hypocotyls should be approx 3- to 7-mm in length and quite pink in color.
6. Hypocotyls are excised from seedlings after 6 to 7 d of culture, cut into 3- to 6-mm pieces (usually 1–3 explants/seedling) and used in transformation experiments.

3.2. Introduction of Binary Vectors Into Agrobacterium

1. Binaries were transformed into electro-competent *Agrobacterium* by electroporation (*see* **Chapter 3**, Volume 1) and a single colony used to establish suspensions for transformation.
2. Frozen glycerol stocks are prepared by inoculating 10 mL MGL containing 20 μg/mL rifampicin, 50 μg/mL spectinomycin, and 50 μg/mL streptomycin, with a single colony and grown at 28°C for 2 nights. The agrobacterial suspension is then diluted with 1 volume of 60% sterile glycerol solution (to make a final glycerol concentration of 30%), mixed thoroughly, and left at room temperature overnight. Aliquots of 0.5 mL are frozen at –80°C.

3.3. Agrobacterium *Transformation of Hypocotyl Explants*

3.3.1. Co-cultivation of Hypocotyl Explants With A. tumefaciens

1. Day 1: Prepare an *Agrobacterium* culture in a sterile 50-mL Falcon tube or sterile 125-mL glass flask by adding 100 μL frozen glycerol stock to 10 mL MGL medium. Grow overnight at 28°C with shaking (approx 200rpm).
2. Day 2: Prepare the *Agrobacterium* suspension by diluting the overnight culture to OD_{600nm} approx 0.25 (equivalent to 5×10^8 cells/mL). Place 10 to 15 mL of the suspension into small Petri dishes (55-mm diameter).
3. Using a sharp scalpel blade excise hypocotyls from seedlings just behind the point where cotyledons emerge, and also at the base of the hypocotyl, close to the root (*see* **Note 2**).
4. Immerse batches of about 50 explants completely in the *Agrobacterium* suspension for 10 to 15 min. Agitate occasionally.
5. Transfer explants directly to CM.
6. Repeat this procedure for 100 to 300 explants (up to 300 explants can co-cultivate on a single CM dish) (*see* **Note 3**).
7. Co-cultivate explants for 2 to 3 d in light at 24°C.

3.3.2. Inhibition of A. tumefaciens *Growth*

1. Day 4 or 5: There should be visible *Agrobacterium* growth around explants after 2 to 3 d co-cultivation and hypocotyls may be slightly swollen.
2. Remove all the explants to a petri dish containing between 20 and 25 mL in sterile distilled water. Agitate for 30 to 60 s and remove water.
3. Repeat step 1 two more times, or until water appears clear of *Agrobacterium*.
4. Place 1 to 2 sheets of sterile filter paper on a Petri dish and gently scoop all the hypocotyls onto this.
5. Separate explants a little to allow thorough blotting and then remove them immediately to CM + selection (150 mg/L timentin and 25 mg/L paromomycin or 10 mg/L phosphirothricin [PPT]). It is important during blotting that explants lose excess moisture but they must not be allowed to dry out completely.
6. Place 10 to 20 explants/dish on CM + selection. Seal dishes with Parafilm and return to culture at 24°C.

3.3.3. Production of Embryogenic Callus Cultures

1. Explants are transferred to fresh CM plus selection agents every 21 d (*see* **Note 4**).
2. Type II callus subsequently forms as small regions of white, compact embryogenic callus. It may appear as early as 4 to 6 wk in control regeneration experiments and generally by about 12 wk for *Agrobacterium*-infected explants.
3. Type II callus is transferred to B50 containing 150 mg/L timentin plus appropriate selection agent and to fresh medium of the same composition every 3 wk.
4. After Type II callus is removed from explants and transferred to B50 plus selection, the explants are also transferred to fresh CM plus selection, where they will potentially develop more Type II callus. This is subsequently transferred to B50 (*see* **Note 5**).

5. At this stage, culture dishes containing Type II callus are transferred to bottom cooling shelves. This bottom cooling greatly facilitates the development of morphologically and physiologically normal plantlets. Our shelves are constructed of 13-mm thick Perspex with internal channels to allow water circulation from one corner to the other, and are fitted onto a standard growth room rack. The surface temperature of the shelf is between 18 and 20°C (*see* **Note 6**).
6. Cultures from each responding hypocotyl piece are numbered and kept separate from each other. In this way the system can be managed to maximise the number of independent transgenic events (*see* **Note 7**).

3.3.4. Development of Somatic Embryos Into Plantlets

1. Somatic embryos may form after one to two culture periods on B50 medium. Plantlets with roots develop from embryos on the same medium and should be allowed to grow to approx 15 to 30 mm in taller culture vessels before being transferred to soil (*see* **Note 8**).
2. Somatic embryos will develop into plantlets at very different rates. Those with normal morphology will develop fastest, whereas some become vitreous with thick, watery leaves. The latter are difficult to culture and may never form normal plantlets. The use of bottom cooling has greatly reduced the frequency of abnormal embryos and plants.
3. When working with shoots remove any basal callus and brown or dead shoots/leaves before transferring to fresh media.

3.3.5. Transfer of Plantlets to Soil

1. Morphologically normal plantlets with good roots are transplanted to 10-cm pots containing soil mix of 50% compost, 50 % Perlite plus Osmocote complete slow release fertilizer.
2. Plants are covered with clear plastic tissue culture pots to maintain humidity. Plants are kept in a growth cabinet under 12 h light at 22°C and 12 h dark at 19°C for 5 to 10 d during which time the covering pot is gradually removed.
3. Plants are transferred to glasshouse and repotted to 20-cm pots containing a premium grade commercial potting mix consisting of composted pine bark, slow release fertilizer, wetting agent, and coir peat.
4. Plants are grown in a glasshouse with d/night temperatures of 25°C/22°C, respectively, and a 16-h d length. Plants are watered daily and fertilized with slow release fertilizer when repotted to larger pots.
5. Plants generally flower 6 to 8 wk after transfer to soil and self pollination can be assisted by applying pollen to the stigmatic disc of the capsule.
6. Seeds are harvested from the capsules when the plant stem and capsules are completely dried off and seeds can be heard rattling within the capsule (approx 12–16 wk from transfer of plants to soil). Well developed capsules can typically produce thousands of seeds.

3.4. Progeny Testing

These methods can be used to confirm the expression of the *pat* (phosphinothricin acetyl transferase) gene and *npt*II gene when used as the selectable marker.

3.4.1. Testing for Herbicide Resistance In Vitro

1. Progeny seed are surface sterilized and germinated on B50 medium containing 10 mg/L PPT to test for stability of *pat* gene expression and segregation of the transgene.
2. Resistant seed will germinate and form normal seedlings with roots. Susceptible seed will also germinate to fully developed cotyledons and first leaves but then will begin to brown and die. Seedlings are usually assessed 10 to 14 d after germination.

3.4.2. Testing for Herbicide Resistance Using Nondestructive Leaf Painting Assay

1. Plants are grown in flat trays at a density of about 400/m^2 (*see* **Note 9**).
2. For nondestructive assay of the presence of the *pat* gene, 1000 ppm of glufosinate ammonium, prepared by diluting Basta® herbicide, is applied to a small area of leaf using a cotton bud.
3. The treated area is marked with an indelible marker pen for ease of identification.
4. The treated area of tolerant leaves will remain green while the treated area of susceptible plants will begin to senesce within 4 to 6 d.

3.4.3. Testing for Herbicide Resistance by Herbicide Spray

1. Plants are grown in flat trays at a density of about 400/m^2 (*see* **Note 9**).
2. For destructive segregation test, Basta® herbicide is applied at 3 L/ha (i.e., 600 g PPT/ha) in 100 L/ha, when the plants are at the 4 to 6 leaf stage.
3. The sprayed tolerant leaves will remain green while the susceptible plants will die within 7 to 10 d.

3.4.4. Testing for Paromomycin Resistance Using Nondestructive Leaf Painting Assay

1. Plants are grown in flat trays at a density of about 400/m^2 (*see* **Note 9**).
2. For nondestructive assay of the presence of the *npt*II gene, leaves are painted with a solution of 1% (w/v) paromomycin sulfate and 0.1% (v/v) Tween-20 added as a wetting agent.
3. The treated area of tolerant leaves will remain green while the treated area of susceptible plants will begin to senesce within 5 to 7 d.

4. Notes

1. Alternatively, after the ethanol wash, seeds can be sterilized for 5 min in a solution of 1% zephiran (80% benzalkonium chloride) in 10% ethanol, and then rinsed as described.
2. It is very important that seedling material is at the correct stage of development to provide healthy explants. Seed germination can sometimes be variable. It is best to judge when seedlings are ready for experiments by their appearance and not by the number of days since germination. Using seedlings older than 9 to 10 d is

likely to result in reduced regeneration efficiencies. Do not use long or etiolated seedlings. Hypocotyls shorter than 3 mm may not form callus and are more likely to become overgrown with *Agrobacterium*.

3. For each *Agrobacterium* construct to be transformed into *P. somniferum* plan on initiating a series of 2 to 3 individual experiments each consisting of 100 to 300 explants. This should provide plants for analysis from an adequate number of independent transformation events. Experiments of this size are quite manageable, and in the event of contamination or poor regeneration in a single experiment, damage (and/or time lost) is minimal (as compared to setting up a single experiment of, say, 1000 explants).
4. Explants will begin to turn dark brown or black. This is normal and does not appear to affect the potential to produce embryogenic callus. Explants initially may form loose translucent callus designated Type I callus. This translucent callus is usually colorless in the early stages of culture and becomes browner over time.
5. Explants have the capacity to produce Type II callus over a period of months. We routinely remove Type II callus from the explants 3 to 4 times before discarding the explants. Type II callus produced after this time tends to become more friable and watery, and probably has less embryogenic potential.
6. The bottom cooling shelves are maintained with a 4 to 6 degree difference between ambient and circulating water. It is likely, but unproved, that the positive effect is because of the dryer vessel atmosphere that results from this temperature differential.
7. Our experience is that multiple regenerants from a single hypocotyl piece will include some clones of the one transgenic event and also independent transgenic events.
8. Normal plantlet development from germinated embryos can take anywhere from 4 wk to several mo (which will involve several transfers to fresh media).
9. Plants of any age can be tested by this method, although as they begin flowering, mature leaves begin to senesce, so it is important to choose healthy green leaf tissue to test, and probably wisest to test plants well before flowering. This also applies to the paromomycin resistant tests.

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VIII

Non-Plants

36

Actinomycetes (*Streptomyces lividans*)

Clarence I. Kado and Brian Kelly

Summary

Agrobacterium tumefaciens is best known for its ability to transform plants by delivering the T-DNA that is processed and transferred from the resident Ti plasmid to the recipient plant cells. Less well known is the capacity of this Gram-negative bacterium to transfer its T-DNA into fungi and actinomycetes. Procedures are described on the use of the promiscuous T-DNA transfer system of *A. tumefaciens* to transform members of the actinomycetes. Integration of the T-DNA derivatives into the *Streptomyces* chromosome provides opportunities of obtaining valuable mutants of this antibiotic producing soil organism.

Key Words: *Streptomyces*; pUCD2614 high copy *vir* helper plasmid; pUCD2335 plant transformation vector; pUCD5801 *Streptomyces* transformation vector.

1. Introduction

Agrobacterium tumefaciens is best known for its ability to transfer DNA to plants. This Gram-negative, rod-shaped bacterium is equipped with a highly conserved set of genes whose products assemble the transmembrane DNA transfer apparatus. For pathogenesis, the T-DNA processed from the resident Ti plasmid is transferred and integrated into the genome of the host plant cells. *A. tumefaciens* is less known for transferring DNA into organism besides plants. For example, various fungi such as yeasts (e.g., *Saccharomyces cerevisiae [1,2]*, *Schizosaccharomyces pombe [3]*, and *Kluyveromyces lactis [4]*), including members of the filamentous *Basidiomycetes (5,6)* are also transformed by *A. tumefaciens*. Moreover, filamentous actinomycetes can also serve as transformable recipients *(7)*. Although transformations of *Streptomyces* spp. have been demonstrated using protoplasts *(8)*, and linear DNA *(9)*, transformation efficiencies remained low and the preparation and handling of protoplasts are cumbersome. Conjugative transfer of DNA is more efficient than transformation.

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Conjugative transfer between *Escherichia coli* and *Streptomyces* species have been demonstrated using derivatives of the broad-host-range plasmid RK2 (*10–12*). *A. tumefaciens* harboring a high-copy virulence regulon helper plasmid pUCD2614 also transfers T-DNA to *Streptomyces* species at relatively high frequencies (*13*). This chapter describes the methodology used for transfer and integration of novel genes into *Streptomyces* spp., an Actinomycete and a member of the family Streptomycetaceae that naturally form mycelial filaments and spores (*14*).

2. Materials

2.1. Bacterial Strains and Plasmid Constructs

1. The recombination deficient strain, *A. tumefaciens* LBA4301, Rif^R, Rec^-, was a gift of Dr. Rob A. Schilperoort (State University Leiden, The Netherlands) (*15*) (*see* **Note 1**).
2. Plasmid-free *Streptomyces lividans* 66 strain TK64 (*str-6 pro-2*) was kindly provided by Dr. Stanley Cohen (Stanford University School of Medicine, Stanford, CA).
3. *E. coli* HB101 [F^-, *hsdS*20, (r,m), *recA*13, *ara*-14, *proA2, lacY1, galK2, galT22, metB1, rpsl20, Sm, xyl-5, mtl-1, supE44*] was from Dr. Francisco Bolivar, formerly of University of California, San Francisco (*see* **Note 2**).
4. Plasmid pUCD2614 (*16*): This is a high-copy conjugation-transfer helper plasmid that contains the entire virulence regulon of pTiC58, a high copy mutant *ori*V of pTiC58 (*see* **Note 3**) and *ori*V of ColE1, two partitioning genes from pTAR (*see* **Note 4**), a neomycin phosphotransferase gene, and an ampicillin resistance gene. This plasmid increases efficiency of T-DNA transfer from *A. tumefaciens* strains (*16,17*).
5. Plasmid pUCD2335: Contains the origin of replication from broad-host-range plasmid pSa, kanamycin, and gentamicin resistance genes and the left T-DNA border and a nopaline synthase promoter and a multiple cloning site (*17*).
6. Plasmid pUCD5801: This is derived from pUCD2335 and pSET152, and contains an integration (*int*) site of phage C31, *ori*Vs of pSa and ColE1, and resistance genes for kanamycin, gentamicin, and apramycin (*13*).

2.2. Culture Media and Growth Conditions

1. 1 L Medium 523 (*18*): *A. tumefaciens* LBA4301 and derivatives are grown on medium 523 agar (15 g Bacto agar/L) and 523 broth at 28°C. Add 10 g sucrose, 8 g tryptone (Difco), 4 g yeast extract, 3 g KH_2PO_4, and 0.3 g $MgSO_4·7H_2O$, pH 7.2.
2. 1 L Vir regulon induction medium (I medium) (*19*): Murashige minimal organics medium (Sigma, St. Louis, MO, cat. no. M6899) supplemented with 12.5 m*M* KH_2PO_4, pH 5.5, and 100 μ*M* acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone) (Aldrich, Milwaukee, WI cat. no. D13440-6).
3. 1 L Luria-Bertani (LB) broth and agar for *E. coli* strains: Add 10 g Tryptone (Difco), 5 g yeast extract, 10 g NaCl, pH 7.2. For solid medium, add 15 g Bacto agar/L.

4. 1 L Yeast extract-malt extract (YEME) medium for *Streptomyces lividans* strains: 340 g sucrose, 10 g glucose, 5 g peptone (Difco), 3 g yeast extract, 3 g malt extract, and 5 m*M* $MgCl_2$.
5. R2 medium *(20,21)*: Contains 80 mL of a solution consisting of 103 g sucrose, 0.25 g K_2SO_4, 10.12 g $MgCl_2 \cdot 6H_2O$, 10 g glucose, 0.1 g casamino acids (Difco), and 800 mL distilled water, add 2.2 g of Bacto-agar (Difco) to 80 mL of this solution and autoclaved. The following are added to the autoclaved solution: 1 mL 0.5% KH_2PO_4; 8 mL of 3.68% $CaCl_2 \cdot H_2O$; 1.5 mL 20% L-proline; 10 mL 5.73% TES (Tris-[hydroxylmethyl]-2-2aminoethane sulphonic acid) buffer, pH 7.2; 0.2 mL trace element solution; and 0.5 mL 1 *N* NaOH. The trace element solution contains 40 mg $ZnCl_2$, 200 mg $FeCl_3 \cdot 6H_2O$, 10 mg $CuCl_2 \cdot 2H_2O$, 10 mg $MnCl_2 \cdot 4H_2O$, 10 mg $Na_2B_4O_7$, and 10 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ per mL.
6. 1 L Pre-germination medium (PG medium): Add 10 g Tryptone (Difco) and 10 g yeast extract. $CaCl_2$ was added to a final concentration of 10 m*M* after autoclaving (to avoid precipitate formation).

2.3. Other Solutions, Reagents, and Supplies

1. TMES buffer: 0.5 *M N*-Tris-[hydroxylmethyl]-methyl-2-aminoethane sulfonic acid, pH 7.0.
2. 1 *M* Acetosyringone: Dissolve the chemical in fresh dimethylsulfoxide as a 1 *M* solution, filter-sterilize, and stored at 4°C. It is added to the autoclaved and cooled medium. Induction was performed at 20°C.
3. Phosphate buffer is 10 m*M* sodium phosphate, pH 7.2.
4. Gentamicin is freshly prepared as a 10 mg/mL stock solution. Stored at 4°C.
5. Chloramphenicol is freshly prepared as a 500 mg/mL stock solution. Store at 4°C.
6. Nalidixic acid is freshly prepared as a 50 mg/mL stock solution and store at 4°C.
7. Apramycin is freshly prepared as a 10 mg/mL stock solution. Stored a 4°C.
8. 1 mL Lysis buffer: 50 µg of freshly prepared lysozyme, 50 µg of DNase-free pancreatic RNase; 0.3 *M* sucrose; 25 m*M* Tris-HCl, pH 8.0; 25 m*M* Na_2EDTA.

3. Methods

3.1. Preparation of Streptomyces Competent Spores

1. *Streptomyces lividans* and related species are grown in YEME broth with rotary shaking at 200 rpm.
2. The filamentous hyphae are disrupted by vortexing briefly and 0.5 mL of the suspension spread onto R2 soft agar medium. Plates are incubated at 28°C for 7 to 8 d for maximum sporulation.
3. Spores are scraped from the agar surface, suspended in 0.5 mL (usually ca. 10^7 spores/mL) of 0.5 *M* TMES buffer and collected by centrifugation at 10,000*g*, for 3 min. The spore pellet is re-suspended in 200 µL 0.5 *M* TMES buffer.
4. The spore suspension is heat shocked in a water bath at 50°C for 10 min and then quickly cooled in crushed ice water to 23°C.
5. The spores are transferred to 2.0 mL of PG medium and incubated at 30°C for 2 to 3 h (without agitation) to allow them to germinate.

6. The freshly germinated spores are collected by centrifugation at 3000*g* for 3 min and washed (*see* **Note 5**). Re-suspend in I medium and use within 20 min.

3.2. Preparation of A. tumefaciens Donor

1. *A. tumefaciens* harboring pUCD2614 and either pUCD5801 or pUCD2335 is streaked on medium 523 agar, incubated 48 h at 28°C. A well isolated single colony is transferred to medium 523 broth containing 20 µg/mL kanamycin and incubated with shaking 200 rpm at 28°C overnight. The culture is diluted to A_{600} of 1.5. *A. tumefaciens* (10^7 cfu) containing high copy *vir* helper plasmid pUCD2614 and either pUCD5801, or pUCD2335 is induced in I medium containing 100 µ*M* acetosyringone (AS) by growth at 20°C for 12 h.
2. Collect the induced cells by centrifugation 5000*g* for 5 min and resuspend the cells in fresh I medium to an final A_{600} of 1.5. The cell suspension is used within 20 min.

3.3. Transformation

1. Mix equal parts (200 µL each) of freshly germinated *Streptomyces* spore suspension (ca. 10^8 spores) with the induced *A. tumefaciens* (pUCD2614, pUCD5801 or pUCD2335) cells.
2. Place the cell mixture onto a cellulose nitrate filter disk set on fresh R2 agar medium containing 100 µ*M* AS.
3. Incubate the Petri plates for 2 d at 20°C.
4. Re-suspend the cells off the filter by vortexing in 0.5 mL of phosphate buffer.
5. Spread evenly 100 µL of the cellular mixture onto R2 medium containing 500 µg/mL gentamicin (at high concentrations gentamicin selects *Streptomyces* transformants).
6. Incubate at 20°C for 4 to 5 d.
7. Wash any residual *Agrobacteium* cells off the agar by washing several times with an aqueous mixture (about 8 mL) of chloramphenicol (500 µg/mL) and 50 µg/mL nalidixic acid (*see* **Note 6**).
8. Air dry the plates uncovered in a laminar flow hood (1–2 hr).
9. Cover the plates and incubate at 30°C for 3 wk.
10. Collect carefully without disturbing the agar the spores with a wire loop and suspended in 5 mL of distilled water (*see* **Note 7**).
11. Spread evenly one-tenth volume of the suspension onto R2 agar medium containing 500 µg/mL chloramphenicol, 500 µg/mL gentamicin, and 500 µg/mL apramycin (for counter selection).
12. Incubate the plates at 30°C for 5 to 7 d. Transconjugants appearing on the agar medium are purified and maintained on R2 medium containing the above antibiotics.

3.4. Selection

1. Examine the agar plates for individual colonies.
2. With sterile tooth picks, transfer several potential transconjugants on to fresh R2 agar plates (plates are placed over quadrille paper as a guide for colony place-

ment), and containing 500 μg/mL chloramphenicol, 500 μg/mL gentamicin, and 500 μg/mL apramycin. Incubate the plates at 30°C for 6 to 7 d.

3. Purify the transconjugants by plating onto fresh R2 selection agar after each growth cycle (6–7 d).
4. Maintain the transconjugants on R2 medium containing 50 μg/mL gentamicin.

3.5. Analysis and Confirmation

Plasmid integration into the chromosome is confirmed by Southern blot analysis of total DNA.

1. Steptomyces mycelia are collected by centrifugation (10,000*g* for 10 min at 4°C).
2. The mycelial pellet is resuspended in 1.0 mL of lysis buffer. The suspension is incubated for 30 min at 30°C.
3. Add 1.0 mL of 2% sodium lauryl sulfate; mix by vortexing for 1 min.
4. Add 1.0 mL of phenol-chloroform-isoamyl alcohol mixture (1:1:0.1 [v:v:v]). Mix by capping and shaking vigorously for 20 s.
5. Centrifuge the mixture at 10,000*g* for 5 min at 4°C. Collect the upper aqueous phase with a wide-mouth pipet to avoid shearing DNA, and extract the aqueous phase with an equal volume of phenol-chloroform-isoamyl alcohol by gently rocking the tube several times until the white interface has been removed.
6. Add 0.1 volume of 3 *M* sodium acetate, pH 5.0, and mix by gently rocking the tube.
7. Add an equal volume of 95% ice cold ethanol to flocculate the DNA, which is collected by centrifugation at 10,000*g* for 15 min at 4°C.
8. The DNA was resuspended in 0.5 mL 0.01 *M* Tris-EDTA buffer, pH 8.0, containing 25 μL 100 m*M* spermine-HCl. Incubate the mixture for 5 min at 23°C.
9. Collect the DNA by centrifugation at 10,000*g* for 15 min at 4°C. Resuspend the clear pellet of DNA in 300 μL 0.3 *M* sodium acetate, pH 4.8, containing 10 m*M* magnesium chloride.
10. Add 700 μL of 95% ethanol. The DNA precipitate is collected by centrifugation, 10,000*g* for 10 min at 4°C, and resuspended in 500 μL TE buffer, pH 8.0.
11. The *Streptomyces* chromosomal DNA can be probed with the appropriate modified T-DNA region of pUCD5801 and pUCD2335. They both contain a 534-bp T-DNA region bearing the nopaline synthase (*nos*) promoter. This region can be amplified by polymerase chain reaction using primers 5′-ACGAGCCAAG GGATCTT-3′ and 5′-GGTAATTGCCATTGCAG-3′.
12. The DNA is labeled with [^{32}P]-dCTP using RadPrime DNA labeling system (Invtrogen, Carlsbad, CA; Cat.no.18428-011). The labeled DNA is used to probe total *Streptomyces* chromosomal DNA by Southern blot hybridization *(24)* (*see* **Note 8**).

4. Notes

1. A recombination deficient strain is used to harbor the *vir* helper plasmid, pUCD2614 and the T-vector plasmid as independent replicons.
2. *E. coli* HB101 is used to harbor pUCD2614 or pUCD5801, and serves as the reservoir for these plasmids.

3. pUCD2614 contains an *oriV* copy control mutation *(22)* to elevate its copy number to at least 6 to 10 plasmid copies/cell. Increased copy number increases plasmid vector T-DNA transfer efficiency *(17)*.
4. Two partitioning (*par*) loci derived from pTAR *(23)* were incorporated into pUCD2614 to stabilize the helper plasmid for prolong maintenance.
5. *Streptomyces* species naturally produce the blue-pigmented antibiotic actinorhodin on certain complex media such as L agar and MS agar, which can inhibit or reduce the growth of *Agrobacterium*. Thorough washing is therefore essential.
6. Mycelial outgrowth of *Streptomyces* germinated spores anchor naturally into the soft agar and are therefore retained even after the washing step. Chloramphenicol and nalixidic acid select against any residual *Agrobacterium* cells. *Streptomyces* is naturally resistant to these antibiotics.
7. Care must be taken to avoid grazing the agar surface too extensively to avoid picking up mucoidal material that will interfere with plating the spores evenly.
8. It is not known how T-DNA is integrated. There are two hypothesis for its integration into large chromosomal DNA: (1) integration by illegitimate recombination or (2) integration at short interspersed repetitive element (SINE) sites by homologous recombination. Vector pUCD5801 contains the integration site (*int*) of phage C31.

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Filamentous Fungi (*Magnaporthe grisea* and *Fusarium oxysporum*)

Chang Hyun Khang, Sook-Young Park, Hee-Sool Rho, Yong-Hwan Lee, and Seogchan Kang

Summary

A better understanding of fungal biology will facilitate judicious use of beneficial fungi and will also advance our efforts to control pathogenic fungi. Molecular studies of fungal biology have been greatly aided by transformation-mediated mutagenesis techniques. Transformation via nonhomologous integration of plasmid DNA bearing a selectable marker (e.g., antibiotic resistance gene) has been widely used for the random insertional mutagenesis of fungi—as an alternative to chemical and radiation mutagens—mainly because the integration of plasmid into the genome provides a convenient tag for subsequent identification and isolation of the mutated gene. Homologous recombination between a target gene on the chromosome and the introduced DNA carrying its mutant allele results in targeted gene knock-out. An important advance in fungal transformation methodology is the development of *Agrobacterium tumefaciens*-mediated transformation (ATMT) protocols for fungi. ATMT has been successfully applied to a phylogenetically diverse group of fungi and offers a number of advantages over conventional transformation techniques in both the random insertional mutagenesis and targeted gene knock-out. In this chapter, we describe ATMT protocols and vectors for fungal gene manipulation using two plant pathogenic fungi, *Magnaporthe grisea* and *Fusarium oxysporum,* as target organisms.

Key Words: *Agrobacterium tumefaciens*; binary vector; conditional negative selection; *Fusarium oxysporum*; gene knock-out; herpes simplex virus thymidine kinase; *Magnaporthe grisea*; transformation.

1. Introduction

Transformation-mediated forward and reverse-genetic analyses have greatly facilitated functional studies of fungal genes *(1)*. In most filamentous fungi, transformation results from the integration of introduced DNA into the fungal genome by either nonhomologous or homologous recombination. Mutagenesis

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of fungi through random integration of the transforming DNA via nonhomologous recombination has been widely used for tagging and isolating fungal genes that are involved in various aspects of fungal biology *(2–5)*. Mutagenesis of a specific gene via homologous recombination between the target gene and its mutant allele that has been introduced through transformation is also routinely performed in many fungi. A typical procedure for transforming filamentous fungi involves the preparation of fungal protoplasts, delivery of the transforming DNA with associated selectable marker, and selection of the generated transformants. The protoplast generation step, which involves the digestion of the fungal cell walls using a mixture of hydrolytic enzymes, is often critical for high-transformation efficiency and is difficult to optimize for reproducibly producing good protoplasts. *Agrobacterium tumefaciens*–mediated transformation (ATMT) offers an alternative and versatile means for transforming fungi without relying on protoplasts.

Since the demonstration of successful T-DNA transfer into *Saccharomyces cerevisiae* by *A. tumefaciens* *(6)*, ATMT has been successfully applied to transform a diverse array of fungi *(7–24)*. For successfully transforming fungi, *A. tumefaciens* appears to utilize the same virulence genes that are required for plant transformation *(6,25)*, suggesting that the bacterium utilizes a conserved mechanism for transformation regardless of hosts. In addition to the ability to transform spores, hyphae, and even mushroom fruiting body tissue, ATMT exhibits a number of other advantages as a method for forward and reverse genetic analyses of fungi: (1) high transformation efficiency, resulting in several hundred transformants per 1×10^6 spores in many fungal species *(12,19,20)*, (2) increased frequency of homologous recombination, a feature conducive for efficient targeted gene knock-out *(13,24,26)*, and (3) low-copy-number of inserted T-DNA per genome (less than two on average), which facilitates the identification of a gene tagged by the T-DNA especially in asexual fungi *(12,19–21)*.

Although targeted gene knock-out via transformation is feasible in many fungi, for fungi that exhibit low frequencies of homologous recombination, identification of the desired mutant often requires that a large number of transformants be generated and screened *(14,27)*. To circumvent this time-consuming process, a new targeted gene knock-out method, which is based on ATMT with a mutant allele of the target gene flanked by a conditional negative selection marker, was developed (*see* **Fig. 1**). A dual (positive and negative) selection of transformants permitted the enrichment of target mutants *(14,27)*. This method, termed ATMT-DS, can potentially be applied to a broad spectrum of fungi and may serve as a powerful functional genomic tool in fungal research. In this chapter, protocols and vectors for ATMT and targeted mutagenesis of filamentous fungi are described using two plant pathogenic fungi, *Magnaporthe grisea*

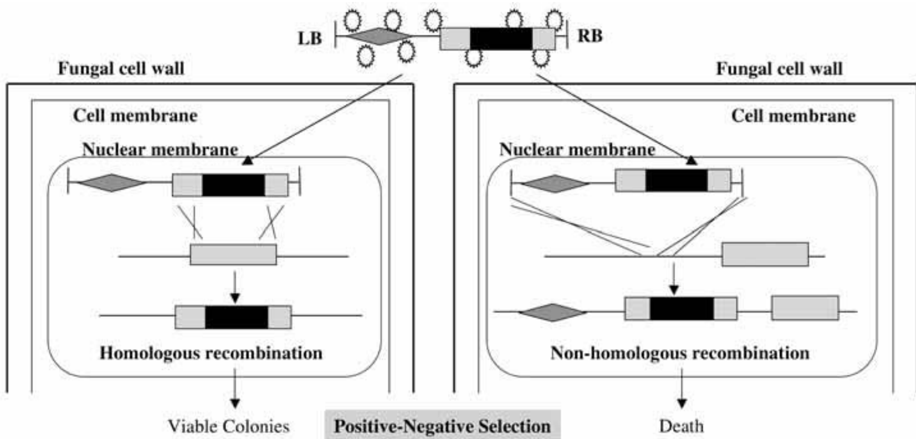

Fig. 1. Schematic diagram of ATMT of fungi and ATMT-DS. *Agrobacterium tumefaciens* cells, carrying a binary vector that contains a mutant allele (disrupted by a positive selection maker, such as *hpt* conferring resistance to hygB; marked as the filled box) and *HSVtk* (encoding herpes simplex virus thymidine kinase that converts nucleoside analogs such as 5-fluoro-2′-deoxyuridine [F2dU] to a compound toxic to fungi; denoted by the diamond) on the T-DNA, are co-incubated with fungal cells in the presence of AS, a chemical inducer of virulence genes of *A. tumefaciens*. During co-cultivation, DNA situated between the left border (LB) and right border (RB) of the T-DNA is transported into fungal nuclei (probably as a complex with certain *A. tumefaciens* Vir proteins which were denoted by the circles). Homologous recombination between the chromosomal gene and the mutant allele on the T-DNA leads to the loss of *HSVtk*. If the T-DNA integrates into a random location in the fungal genome via non-homologous recombination, both *hpt* and *HSVtk* will be expressed. Gene knock-out mutants can be selected by subjecting transformants to both the positive (hygB) and negative (F2dU) selection agents.

and *Fusarium oxysporum*, as target organisms. In both fungi, we typically obtain between 100 and 400 transformants per 1×10^6 spores. These tools can also be applied to other fungi with no or little modification.

2. Materials

2.1. Fungal and Bacterial Strains (see *Note 1*)

1. *M. grisea* KJ201 ***(28)***: An isolate from infected rice.
2. *M. grisea* 4091-5-8 ***(29)***: A laboratory strain derived from a genetic cross between two field isolates.
3. *F. oxysporum* strain O-685 ***(19)***: An isolate from cabbage.
4. *A. tumefaciens* strains AGL1 and EHA105 ***(30)***.
5. *Escherichia coli* strain XL1-Blue MRF (Stratagene, La Jolla, CA).

2.2. Culture Media

All culture media use 1.5% agar (Difco, Sparks, MD) to solidify medium and are autoclaved (120°C for 15 min) before use.

1. Oatmeal agar: After incubating 50 g of rolled oats (Quaker Oats, Chicago, IL) in 500 mL at 70ºC for 1 h, filter them through a cheesecloth, add agar, and bring the volume to 1 L.
2. Complete medium: 6 g of yeast extract, 6 g of casein acid hydrolysate, 10 g of sucrose/L.
3. 2YEG: 2 g of yeast extract and 10 g of glucose/L.
4. Carnation leaf agar: After sterilizing 1.5% agar in water by autoclaving, cool to 60°C. Pour agar solution in 90 mm diameter Petri plates containing sterilized (via irradiation) carnation leaf pieces (20–30 pieces/plate) (*see* **Note 2**).
5. Potato dextrose: 24 g of potato dextrose/L (Difco, Sparks, MD).
6. *Aspergillus* minimal medium: For 1 L of medium, add 50 mL stock salt solution (60 g $NaNO_3$, 5.2 g KCl, 5.2 g $MgSO_4 \cdot 7H_2O$ and 8.2 g KH_2PO_4/L), 10 g glucose, and 2 mL Hutner's trace elements (2.2 g $ZnSO_4 \cdot 7H_2O$, 1.1 g H_3BO_3, 0.5 g $MnCl_2 \cdot 4H_2O$, 0.5 g $FeSO_4 \cdot 7H_2O$, 0.16 g $CoCl_2 \cdot 6H_2O$, 0.16 g $CuSO_4 \cdot 5H_2O$, 0.11 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ and 5 g Na_2EDTA/100 mL).
7. CMC broth: 15 g of carboxymethyl cellulose, 1 g of yeast extract, 0.5 g of $MgSO_4$, 1 g of NH_4NO_3, and 1 g of KH_2PO_4/L (*see* **Note 3**).
8. Water agar: Autoclave 1.5% agar in water.
9. Luria–Bertani broth (LB): 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl/L.
10. Minimal medium (MM): *See* **Table 1** and **Note 4**.
11. Induction medium (IM): *See* **Table 1** and **Note 5**.
12. Co-cultivation medium (CM): *See* **Table 1** and **Note 5**.

2.3. Antibiotics and Other Chemical Agents (see *Note 6*)

1. Carbenicillin: Prepare as a stock of 100 mg/mL in H_2O.
2. Kanamycin: Prepare as a stock of 50 mg/mL in H_2O.
3. Rifampicin: Prepare as a stock of 10 mg/mL in methanol.
4. Acetosyringone (AS) (3,5-dimethoxy-4-hydroxyacetophenone): Prepare as a stock of 200 m*M* in 95% ethanol (*see* **Note 7**).
5. Hygromycin B (hygB): Prepare as a stock of 100 mg/mL in H_2O.
6. Cefotaxime, sodium salt: Prepare as a stock of 200 m*M* in H_2O.
7. Moxalactam, sodium salt: Prepare as a stock of 100 mg/mL in H_2O.
8. F2dU (5-fluoro-2′-deoxyuridine): Prepare as a stock of 10 m*M* in H_2O.
9. 1 M MES: dissolve 42.64 g of MES [2-(*N*-morpholino)ethanesulfonic acid] in 160 mL of deionized H_2O. The pH is adjusted to 5.3 with 10 *N* NaOH with stirring vigorously on a magnetic stirrer until the MES has completely dissolved. Then, deionized H_2O is added to bring the final volume to 200 mL (*see* **Note 8**).

2.4. Binary Vectors for Fungal Transformation (see *Note 9 and Fig. 2*)

1. pBHt2: A vector carrying the *hpt* (hygB phosphotransferase) gene under the control of the *Aspergillus nidulans TrpC* promoter on the T-DNA.

Table 1
Preparation of Stock Solutions and Transformation Media

	Stock solution (100 mL)[a]		MM[b]	IM[c]	CM[d]
Reagent	Chemical	Amount required	Amount required to make 100 mL		
K-buffer (pH 7.0)	K_2HPO_4	20 g	1 mL	1 mL	1 mL
	KH_2PO_4	14.5 g			
M-N	$MgSO_4{\cdot}7H_2O$	3 g	2 mL	2 mL	2 mL
	NaCl	1.5 g			
1% $CaCl_22H_2O$	$CaCl_2{\cdot}2H_2O$	1 g	0.1 mL	0.1 mL	0.1 mL
Spore elements	$ZnSO_4{\cdot}7H_2O$	0.01 g	1 mL	1 mL	1 mL
	$CuSO_4{\cdot}5H_2O$	0.01 g			
	H_3BO_3	0.01 g			
	$MnSO_4{\cdot}H_2O$	0.01 g			
	$Na_2MoO_4{\cdot}2H_2O$	0.01 g			
20% NH_4NO_3	NH_4NO_3	20 g	0.25 mL	0.25 mL	0.25 mL
20% Glucose	Glucose	20 g	1 mL	1 mL	1 mL
0.01% $FeSO_4$	$FeSO_4$	0.01 g	1 mL	1 mL	1 mL
50% Glycerol	Glycerol	50 mL	–	1 mL	1 mL
1M MES (pH 5.3)	MES	21.32 g	–	4 mL	4 mL
Sterile H_2O			93.5 mL	88.5 mL	88.5 mL
Kanamycin stock			0.15 mL	0.15 mL	0.15 mL
Acetosyringone			–	0.2 mL	0.2 mL
Agar			–	–	1.5 g

[a]Prepare all the stock solutions (except AS) in deionized H_2O as indicated. It is not necessary to sterilize the acetosyringone stock. Glucose, $FeSO_4$, kanamycin, and MES are sterilized by filtration through a 0.22-µm filter, and the remaining reagents are sterilized by autoclaving for 20 min at 120°C. Store them at 4°C except $FeSO_4$, kanamycin, acetosyringone, and MES (stored at –20°C).

[b]Minimal medium (*see* **Note 4**).

[c]Induction medium (*see* **Note 5**).

[d]Co-cultivation medium. CM is identical to IM except the addition of 1.5% agar.

2. pKHt: A vector carrying the *hpt* gene under the control of the *A. nidulans TrpC* promoter plus the ColE1 replication of origin and the chloramphenicol resistance gene on the T-DNA.
3. pDHt: A vector for the targeted mutagenesis of fungal genes via homologous recombination.
4. pGKO1: A vector for the targeted mutagenesis of fungal genes via ATMT-DS.
5. pGKO2: A vector for the targeted mutagenesis of fungal genes via ATMT-DS.

2.5. Other Solutions and Supplies

1. 2-mL Cryostorage tubes.
2. 80% sterilized glycerol.

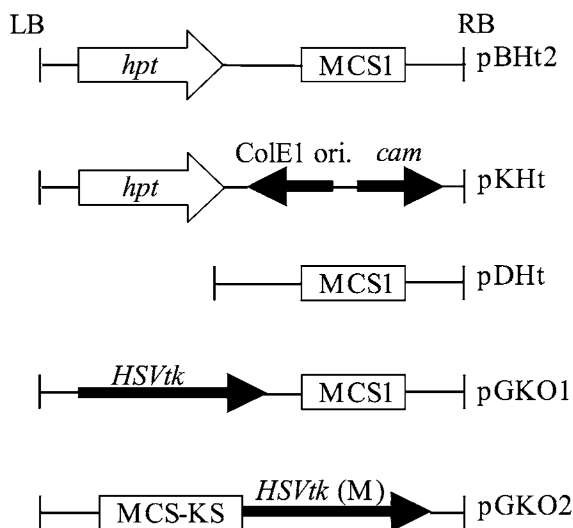

Fig. 2. Schematic diagrams of the T-DNA region in five binary vectors for fungal transformation. The LB and RB of the T-DNA are denoted by vertical lines. The orientation of transcription from *hpt, cam* (conferring resistance to chloramphenicol), and *HSVtk* is indicated by arrow (5′ to 3′). ColE1 ori indicates the ColE1 replication origin. MCS1 corresponds to the multiple cloning site of pCAMBIA1300 (offering nine unique restriction sites in pBHt2 and pDHt). In pGKO1, due to the presence of one or more of the *Bam*HI, *Pst*I, *Sac*I, *Sal*I, and *Sma*I site in *HSVtk*, only four restriction sites in MCS1 can be used for cloning a mutant allele. The multiple cloning site of pGKO2 (designated as MCS-KS) was derived from pGreenII0000 *(34)* and offers 15 unique restriction sites. The *Bam*HI, *Eco*RV, *Pst*I, *Sac*I, *Sal*I, and *Sma*I sites that are present in *HSVtk* were removed via site-directed mutagenesis to produce *HSVtk* (M). Drawings are not to scale.

3. 3-mm Sterilized soda lime glass beads (*see* **Note 10**).
4. Hemocytometer.
5. Cheesecloth.
6. 47-mm Nitrocellulose membrane.
7. Pasteur pipet (melt the tip in flame to produce a round bulb).
8. Genomic DNA extraction buffer: 50 m*M* Tris-HCl, pH 7.5; 100 m*M* ethylenediamine tetraacetic acid (EDTA); 0.5 % SDS; 300 m*M* sodium acetate, pH 6.
9. 10 mg/mL Proteinase K.
10. TE saturated phenol:chloroform (1:1).
11. Isopropanol.
12. 70% ethanol.
13. 10 mg/mL RNase A.
14. 1X TE buffer: 10 m*M* Tris-HCl, pH 7.5 and 1 m*M* EDTA.
15. Ethidium bromide: Prepare as a stock of 10 mg/mL in 1X TE.

16. DNA labeling kit.
17. 0.2 mL thin-wall polymerase chain reaction (PCR) tubes.
18. Taq DNA polymerase and reaction buffer.

3. Methods

3.1. Transformation of A. tumefaciens *With Binary Vector (*see also *Chapter 3, Volume 1)*

3.1.1. Preparation of Competent Cells

1. Streak *Agrobacterium* cells stored in 20% glycerol stock at –80°C on LB agar plate amended with appropriate antibiotic (30 μg/mL of rifampicin for strain EHA105 and 100 μg/mL of carbenicillin for strain AGL1).
2. Incubate the plate at 25 to 28°C until colonies appear (about 2 d).
3. Inoculate a single colony into 4 mL LB containing appropriate antibiotic and grow at 28°C, 250 rpm to log phase (OD_{600} = 0.3–0.6).
4. Inoculate the 4 mL culture into 100 mL LB in a 500 mL flask and grow at 28°C, 250 rpm to OD_{600} = 0.5.
5. After chilling the culture for 10 min on ice, pellet *Agrobacterium* cells by centrifugation at 3000*g* for 10 min at 4°C.
6. Remove LB and resuspend the cells with 2 mL of 20 m*M* $CaCl_2$.
7. After dispensing cells into 1.5 ml microcentrifuge tubes (100–200 μL/tube), freeze them in liquid nitrogen and store at –80°C until needed.

*3.1.2. Transformation (*see *Note 11)*

1. Add 1 μg of vector DNA (in about 5 μL) in 1.5-mL microcentrifuge tube.
2. Thaw competent cells on ice.
3. Dispense 50 μL of competent cells into each microcentrifuge tube containing DNA and mix them by gently pipetting up and down 2 to 3 times.
4. Freeze in liquid nitrogen for 5 min (*see* **Note 12**).
5. Heat shock the frozen cells at 37°C for 20 min.
6. After adding 0.7 mL of LB, grow cells at 28°C at 250 rpm for 2 h.
7. Spread cells on LB agar plate containing 75 μg/mL kanamycin (for all the vectors described in **Subheading 2.4.**) using glass beads (*see* **Note 10**) and incubate the plates (upside down) at 28°C until transformant colonies appear (about 1.5–2 d).
8. Pick two independent transformants and culture them in MM amended with 75 μg/mL kanamycin (*see* **Note 13**).

3.2. Transformation of M. grisea *and* F. oxysporum

3.2.1. Preparation of M. grisea *Spores*

1. Inoculate *M. grisea* on oatmeal agar and grow for 1 to 2 wk under constant fluorescent light at room temperature.
2. Harvest *M. grisea* spores by scraping fungal culture with 1-mL micropipet tip after flooding the plate with 10 mL of sterile water.

3. Filter the spore suspension through two layers of cheesecloth to remove large debris.
4. Determine spore concentration using a hemacytometer. Adjust the spore concentration to 10^6 spores/mL (*see* **Note 14**). If the original solution is too diluted, concentrate them by resuspending with a smaller volume of water after centrifugation.

3.2.2. Preparation of F. oxysporum Spores

1. Inoculate *F. oxysporum* spores to CMC broth and culture on a rotary shaker (100 rpm) at room temperature for 1 wk (*see* **Note 15**).
2. Filter the culture through two layers of cheesecloth to remove mycelia.
3. Harvest spores by centrifugation at 3000*g* for 5 min followed by 2 washes with sterile water.
4. Resuspend spores in sterile water and adjust the spore concentration to 10^6 spores/mL (*see* **Note 14**).

3.2.3. Transformation

1. Incubate the *Agrobacterium* strain containing a binary vector described in **Subheading 2.4.** in 1 mL MM amended with 75 μg/mL kanamycin at 28°C at 250 rpm for 2 d.
2. The *Agrobacterium* cells are diluted to OD_{600} = 0.15 in IM amended with kanamycin and 200 μ*M* acetosyringone (AS) and incubate for an additional 6 h at 28°C at 250 rpm (OD_{600} reaches around 0.6).
3. Mix 100 μL of fungal spore suspension with 100 μL of *Agrobacterium* cells in a microcentrifuge tube and spread the mixture on a nitrocellulose membrane placed on CM in a small Petri plate (15 × 50 mm) (*see* **Note 16**).
4. Incubate the plate for 2 d at 25°C in dark (*see* **Note 17**).
5. Transfer the membrane onto appropriate selection medium amended with hygB for selecting fungal transformants. For *M. grisea*, use complete medium agar amended with 250 μg/mL hygB, 200 μ*M* cefotaxime, and 100 μg/mL moxalactam. *Aspergillus* minimal medium amended with 75 μg/mL hygB, 200 μ*M* cefotaxime, and 100 μg/mL moxalactam is used for selecting *F. oxysporum* transformants (*see* **Note 18**).
6. Incubate the plate at 25°C until hygB-resistant colonies appear (*see* **Note 19**).

3.3. Isolation and Purification of Transformants

1. Prepare 24-well microtiter plates that contain appropriate medium for sporulation: oatmeal agar for *M. grisea*, and CMC broth for *F. oxysporum* (*see* **Note 20**).
2. Transfer a little bit of mycelia from individual transformants to microtiter well using a sterile toothpick or a fine tipped forcep and incubate the plate at 25°C (*see* **Note 21**).
3. For *M. grisea*, flood each well with sterile water and pipet up and down to dislodge conidia. *F. oxysporum* culture in CMC requires no treatment.
4. After plating conidia on solid medium (water agar for *M. grisea* and *Aspergillus* minimal medium for *F. oxysporum*) amended with appropriate antibiotics and incubating for 24 h, pick 1 or 2 single germinating spores for each transformant under microscope and transfer them on appropriate culture medium for subsequent analysis and preservation (*see* **Note 22**).

3.4. Molecular Analysis of Transformants

Southern analysis, PCR, or both have been applied for: (1) testing the intactness of a gene (e.g., a reporter gene such as green fluorescent protein [GFP]) introduced via ATMT, (2) determining the copy number of inserted T-DNA, (3) identifying gene knock-out mutants, and (4) isolating genomic regions flanking inserted T-DNA.

*3.4.1. Genomic DNA Extraction and Southern Analysis (*see *Note 23)*

1. Prepare 24-well plates containing 1 mL of liquid medium (2YEG for *M. grisea* and potato dextrose broth for *F. oxysporum*). Given the mitotic stability of inserted T-DNA ***(19,20)***, after purifying transformants through single spore isolation, it is not necessary to add antibiotics in growth medium.
2. Inoculate individual transformants to be analyzed in each well and seal the plate with parafilm to prevent evaporation of medium.
3. Grow the transformants for a week at room temperature (RT) on a rotary shaker set at 100 rpm.
4. Grind the cultures in 24-well plate using a hand-made 24-well format grinder (*see* **Note 24**).
5. After adding 0.6 mL extraction buffer and 0.5 µL proteinase K/well, seal the 24-well plate with aluminum foil tape and mix thoroughly by inverting the plate.
6. Incubate at 65°C for 1 h.
7. Transfer the samples into individual microcentrifuge tubes and add 0.6 mL TE saturated phenol:chloroform (1:1). Mix well by inverting the tubes several times.
8. Centrifuge for 10 min at 12,000*g* at RT to separate the two phases.
9. Transfer the aqueous layer to clean tubes and precipitate DNA by adding 420 µL isopropanol (0.7 volume of the sample) and mixing the content.
10. Centrifuge for 10 min at 12,000*g* at RT to pellet DNA.
11. After discarding supernatant, wash DNA with 70% ethanol and dry.
12. Resuspend DNA in 20 to 40 µL TE. Dissolve pellet by gently tapping the tubes.
13. Treat dissolved DNA with 10 mg/mL of 1 µL of RNase A for 30 min at 37°C.
14. Digest 10 µL of DNA with appropriate restriction enzyme for 2 to 3 h in a total volume of 30 µL (*see* **Note 25**).
15. Run digested DNA on 0.7% agarose gel (*see* **Note 26**) and blot the gel on a hybridization membrane.
16. Hybridize the blot with appropriate probe.

*3.4.2. Identification of Gene Knock-Out (KO) Mutants by PCR (*see *Note 27)*

1. Design a pair of primers, one corresponding to the 3′- or 5′-end of the positive selection marker (hygB resistance gene) and the other corresponding to a target gene. Design another pair of primers specific to a different gene in transformed fungus for testing the quality of DNA template (*see* **Note 28**).

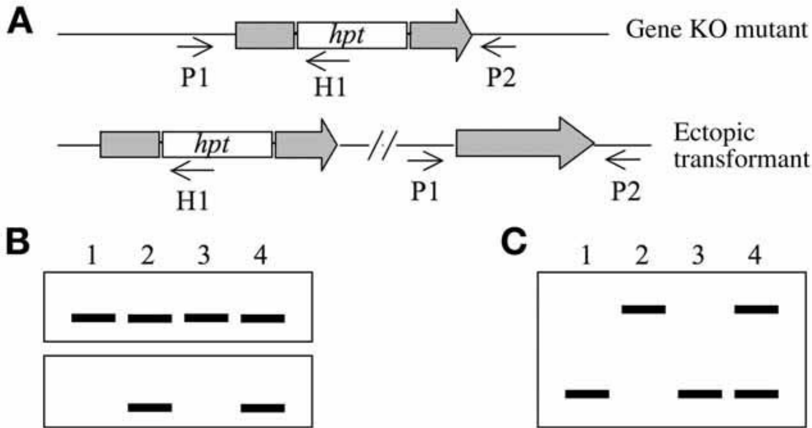

Fig. 3. PCR-based screening of gene KO mutants. **(A)** Schematic diagrams of gene KO mutant and ecotopic transformant. Arrows indicate the PCR primers used for PCR screening. Primers P1 and P2 are designed to anneal to the outside of the gene fragment used for creating a mutant allele, and H1 binds to the positive selection marker *hpt*. **(B,C)** Expected PCR product patterns from four different types of fungal strains: lane 1 (wild-type strain), lane 2 (gene KO mutant), lane 3 (ectopic transformant), lane 4 (transformant that has both gene KO and ectopic integration). **(B)** Top panel shows PCR products generated using a pair of primers for testing the quality of PCR template. Bottom panel shows expected PCR products using the primers P1 and H1. **(C)** Expected PCR products based on the primers P1 and P2.

2. Run PCR under appropriate conditions (e.g., primer annealing temperature, extension time according to the expected size of PCR product).
3. Analyze PCR products by running them on agarose gel. Expected patterns are illustrated in **Fig. 3**.

3.4.3. Thermal Asymmetric Inter-Laced (TAIL) PCR

Random, insertional mutagenesis of the fungal genome via ATMT is an efficient forward genetic tool for isolating mutants that are defective in various aspects of fungal biology. Inserted T-DNA provides a molecular tag for isolating genes mutagenized by insertion. TAIL-PCR can be used to isolate the genomic DNA of fungal transformant flanking the site of T-DNA insertion. This method allowed efficient isolation of the host DNA sequence flanking a site of T-DNA insertion in *F. oxysporum* ***(19)***. Plasmid rescue can also be used as an alternative method to TAIL-PCR (*see* **Note 29**).

1. Synthesize primers binding T-DNA border sequence as well as one or more arbitrary degenerate (AD) primer as shown in **Fig. 4** (*see* **Note 30**).
2. Run PCR reactions as previously described ***(19)***.

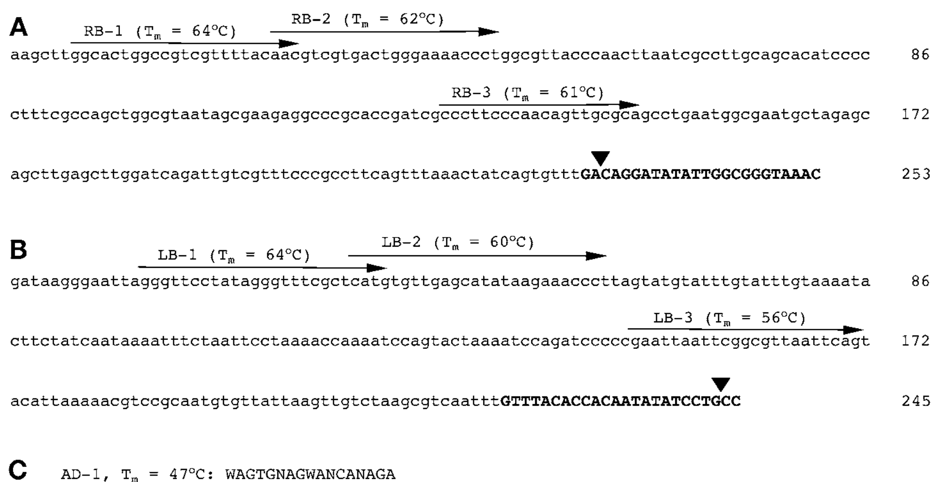

Fig. 4. Right **(A)** and left **(B)** border ends of the T-DNA sequence in the vectors described in **Subheading 2.4**. The position and melting temperature (T_m) of the primers employed for TAIL-PCR were indicated. Bold uppercase letters indicate the 24-bp imperfect repeat (LB and RB) of the T-DNA, which is cleaved at the indicated positions prior to transfer. The sequence and T_m of the degenerate arbitrary primer (AD-1) is also shown **(C)**. In AD-1, W = A or T, and N = A, C, G, T.

3. Separate PCR products on 1% agarose gel to isolate appropriate fragments (*see* **Note 31**).
4. Sequence the isolated fragments using the outermost specific primer, either RB-3 or LB-3, as the sequencing primer.

3.5. Targeted Gene Knock-Out (KO) via ATMT-DS

1. Transform fungal spores as described above (*see* **Subheading 3.2.**), with *Agrobacterium* cells containing a gene KO vector that carries a mutant allele created by inserting the *hpt* gene flanked by the *tk* gene (a conditional negative selection marker conferring sensitivity to F2dU) under the control of a fungal promoter (*see* **Fig. 1**).
2. HygB-resistant transformants are subjected to negative selection by transferring individual transformants using sterilized toothpicks to a selection medium containing 5 μ*M* F2dU in addition to hygB and cefotaxime (*see* **Note 32**).
3. Incubate the transformants for 3 to 5 d at 25°C in dark.
4. Transfer viable transformants to appropriate medium in 24-well microtiter plates for sporulation.
5. Isolate single spores as described above (*see* **Subheading 3.3.**) and analyze purified transformants for the presence of desired mutation (*see* **Note 33**).

4. Notes

1. To ensure phenotypic stability of fungal strains, avoid subculturing them. Repeated subculturing on nutrient-rich medium can potentially lead to loss of virulence and

fertility and reduced conidiation (*29,31*). Thus, it is highly recommended that a fresh culture should be activated from a stock stored at a non-metabolizing state prior to experiment. For long-term storage of *F. oxysporum*, conidia are stored in 15% glycerol at –80°C or fungal cultures grown on carnation leaf agar (*see* **Subheading 2.2.**) are preserved as described in Fisher et al. (*31*) at −20°C. Stock cultures of *M. grisea* are prepared and stored as described by Valent et al. (*29*). After placing sterilized Whatman filter paper disks on oatmeal agar or complete medium plate (*see* **Subheading 2.2.**), fungal culture is allowed to grow over the disks. Once the fungus has completely colonized the disks, the disks are removed and dried. The disks are placed in sterilized post stamp envelope or 2-mL cryo-storage tube and stored at –20°C.

2. Sterilized carnation leaf pieces can be purchased from the *Fusarium* Research Center (FRC) at Penn State (*http://www.cas.psu.edu/Docs/CASDept/Plant/FRC/*; 814-863-0145).
3. It can take several hours for CMC to dissolve completely. While vigorously stirring the solution, add CMC small portion at a time so that it does not form a big clump. Heating shortens the time needed for dissolving CMC.
4. MM (sans $FeSO_4$ and an appropriate antibiotic) can be stored at 4°C for several months. Appropriate amount of 0.01% $FeSO_4$ and an antibiotic are added into MM prior to growing *Agrobacterium* strains.
5. IM (sans $FeSO_4$, MES, an appropriate antibiotic, and AS) can be stored at 4°C for several months. For preparing CM, after autoclaving and cooling down the medium to around 70°C, add 0.01% $FeSO_4$, 1 *M* MES, and acetosyringone as described in Table 1.
6. Prepare antibiotics stock solutions using deionized water and sterilize them by filtering through a 0.22 µm filter. All the antibiotics stock solutions, except hygB (4°C), are stored at −20°C.
7. AS stock solution forms crystals at −20°C. These crystals will quickly dissolve in ethanol when the tube is inverted several times (or briefly vortexted) at room temperature.
8. The filter-sterilized MES stock is stored frozen at −20°C. When the frozen solution is thawed, precipitation appears as white powder. Vortex vigorously until the precipitate has completely dissolved.
9. The vectors described here were derived from pCAMBIA1300 (*http://www.cambia.org/main/r_et_camvec.htm*). Construction scheme for these vectors was previously described (*19,27*).
10. These beads are quite handy for quickly and uniformly spreading bacterial/fungal cells on solid medium, especially when a large number of samples need to be plated (3–4 beads/per 90-mm plate). Wash the beads by soaking them in 1 *N* HCl for several hours. Rinse them with water thoroughly until pH is above 5, dry in a baking oven, and autoclave.
11. Since only one or two *A. tumefaciens* transformants are needed for subsequent step, scaling down the protocol ($^1/_2$ to $^1/_4$) often works fine.
12. Alternatively, freeze cells in –80°C for 15 min.

13. If not used immediately, store these cultures at –80°C until needed as 20% glycerol stock. After fungal transformation, save the one that has yielded higher transformation efficiency as a permanent stock for future use. If one wants to determine whether gene(s) on the introduced binary vector is intact, PCR with primers specific to the target gene can be performed using 1 to 2 μL of intact cells in MM as a template. Alternatively, one can extract plasmid DNA from 5 mL bacterial culture using a commercial silica membrane-based spin column and perform restriction enzyme analysis. Although the yield of plasmid DNA is much lower than that from *E. coli*, we find it sufficient for restriction enzyme analysis.
14. Optimal spore concentration may vary depending on fungal strain/species. The efficiency of transformation via ATMT varied significantly (more than two orders of magnitude) among several fungal species ***(12)***. Even within a species, we noticed significant strain-dependent variation in transformation efficiency ***(19)***. Therefore, determining transformation efficiency for a target strain/species is highly recommended prior to launching a large-scale transformation. The *M. grisea* and *F. oxysporum* strains used here (*see* **Subheading 2.1.**) typically yield 10 and 40 transformants per membrane between (100–400 transformants/1×10^6).
15. Alternatively, culture *F. oxysporum* on carnation leaf agar, which takes much longer than culturing in CMC to produce enough spores for transformation.
16. Use of nitrocellulose membrane does not appear to be essential for successful transformation. The number of plates needed varies depending on the objective of transformation. For generating a pool of insertional mutants randomly tagged by the T-DNA, a large number of plates are needed. However, for introducing a reporter gene (such as the *gfp* gene) to a fungal strain, one plate should be sufficient.
17. Duration of cocultivation is one of the parameters that may require optimization for efficiently transforming a new fungal strain/species. Although transformation efficiency generally increases with the duration of cocultivation in both *F. oxysporum* and *M. grisea* (***19,20***), excessive mycelial growth during prolonged co-cultivation might make it difficult to subsequently identify individual transformants. For certain fungal species such as *Fusarium graminearum*, mycelial growth is rapid and excessive during cocultivation, which appears to inhibit bacterial growth and leads to very low transformation efficiency.
18. Geneticin can also be used for selecting transformants from both species (when transformed with a vector carrying the gene conferring resistance to this antibiotic). Optimal hygB (and geneticin) concentration for selecting transformants may vary significantly from one species to another. Sensitivity to these antibiotics can also vary depending on media used. Prior to transforming a new fungal species via ATMT, one must determine the degree of antibiotic sensitivity of the species by inoculating untransformed strain on several media amended with varying concentrations of chosen antibiotic (a 24-well microtiter plate is suitable for this testing). Use a concentration that completely blocks the growth of untransformed strain for selecting transformants.
19. Bacterial cells frequently form a thick lawn on the membrane after 2 d of cocultivation. Reducing the amount of bacterial cells from the membrane seems to

increase the transformation efficiency and speed up the growth of transformants. Place the membrane upside down on selection medium (90-mm Petri plate) for a few minutes. Drag around the membrane on the surface of the medium to remove bacterial cells as much as possible, and subsequently flip and place the membrane on the same medium. Transformant colonies mainly form on the membrane with colonies appearing occasionally outside of the membrane. It takes approx 4 to 7 d to observe transformant colonies.

20. In addition to *hyg*B (or geneticin), add cefotaxime and moxalactam to the medium to ensure that no *Agrobacterium* cells transferred along with fungal transformants can grow.
21. It may take up to 1 wk to produce conidia from *M. grisea*, whereas 2 to 3 d of culture is often sufficient to produce conidia from *F. oxysporum*.
22. Mix a small volume of conidial suspension (1–3 µL) with a sterilized water drop (100–200 µL) on the surface of medium in small Petri plate (50-mm diameter). Spread conidia using glass beads (*see* **Note 10**). For isolating single spores from a large number of transformants, we use the following method: (1) Prepare a thin layer of medium inside the lid of regular Petri plate (90-mm diameter); (2) draw lines on the plastic surface to divide the medium into 16 sections (number them); (3) touch the culture of transformant with the rounded tip of Pasteur pipet (produced by melting the tip in flame); (4) streak spores attached to the tip on the medium as if plating bacterial cells; (5) after sterilizing the pipet tip, plate another transformant to the next section; (6) Cover the medium with the bottom part of Petri plate and seal with parafilm; (7) grow at 25°C for 24 h, and (8) cut out an agar block containing a single germinating conidium using the flattened tip of a pin (or a platinum wire) under a compound microscope (100 × magnification).
23. This DNA extraction protocol is for quickly analyzing transformants and may not be suitable for analyses requiring a high quality genomic DNA preparation. DNA yield from *F. oxysporum* is significantly lower than that from *M. grisea*, mainly as a result of less mycelial growth under this condition. For more genomic DNA, culture *F. oxysporum* transformants in potato dextrose broth in small Petri plates (after inoculation, leave them on lab bench for 1wk).
24. It is not essential to remove the medium. Alternatively, transfer the cultures into individual microcentrifuge tubes after blotting between paper towels and grind them using a Teflon bar. Freeze-drying is not necessary, but doing so increases DNA yield.
25. For determining the copy number of inserted T-DNA, use a restriction enzyme that has no or one recognition site on the T-DNA construct used for transformation. For checking gene disruption, use a restriction enzyme that has no recognition site on the positive selection marker (e.g., hygB-resistance gene) interrupting the target gene.
26. Before running the gel, run 5 µL of digested DNA in 0.7% agarose minigel to confirm complete digestion. If DNA has not been completely digested, further purify genomic DNA. After increasing the volume of DNA solution to 200 µL with TE, repeat a phenol/chloroform extraction. For DNA precipitation, add 10 µL of 4 *M* NaCl and 400 µL of 100% ethanol. Resuspend DNA in TE.

27. PCR analysis can be performed prior to single spore isolation to reduce the number of transformants that need to be purified. A protocol by Xu and Hamer *(32)* is suitable for quickly analyzing transformants by PCR without purifying genomic DNA.
28. The target gene-specific primer should anneal to the outside of the gene fragment used for creating a mutant allele (*see* **Fig. 3**) so that when combined with a primer specific to the positive selection marker, only gene KO mutants yield a PCR product. The second pair of primers should produce a PCR product from all transformants and serve to test the quality of PCR template. Alternatively, instead of using two pairs of PCR primers, a single pair of primers that bind to the outside of the mutant allele can be used for both purposes. Ectopic transformants should produce a PCR product that corresponds to the wild-type gene, whereas gene KO transformants produce a PCR product that is larger than that from ectopic transformants due to the presence of the positive selection marker. Although this method requires less PCR reactions than the former method, the size of both PCR products to be amplified is larger than that by the former method, thus requiring a longer PCR extension time. Because it also amplifies two PCR products that differ by the size of the positive selection marker (e.g., 1.4 kb for the *hpt* gene), optimizing PCR conditions might be more difficult.
29. If the border sequences become truncated beyond the annealing site for the amplifying borders, PCR amplification of the desired product would not occur. Although the frequency of T-DNA truncation in *F. oxysporum* and *M. grisea* does not appear to be as high as that in plants, such events have been detected. Unless the truncation is extensive, reaching deeply into the T-DNA, plasmid rescue can solve this problem. The binary vector pKHt (see **Subheading 2.1.**) carries the ColE1 replication of origin and the chloramphenicol resistance gene *(19)*. Therefore, from mutants generated using pKHt, inserted T-DNA along with its flanking genomic DNA can be cloned into *E. coli* as a replicating plasmid as follows: (1) digest genomic DNA of selected transformant with a restriction enzyme that does not have a recognition site on the T-DNA, (2) ligate digested DNA, and (3) transform *E. coli* and isolate chloramphenicol resistant colonies. The fungal genes tagged by T-DNA of pKHt can also be identified via TAIL-PCR.
30. We designed a 16-nucleotide (nt) long arbitrary degenerate (AD) primer, labeled AD-1, with a calculated melting temperature (T_m) of 47°C. T_m of primers specific for each border sequence of the T-DNA (LB-1, 2, and 3 for the left border and RB-1, 2, and 3 for the right border), ranging from 20 to 26 nt in size, were designed to be at least 58°C or higher, as previously recommended *(33)*. The 5′-end of LB-3 and RB-3 was 74 bp and 63 bp apart from the 5′-end of LB-2 and RB-2, respectively, to facilitate the identification of PCR products corresponding to T-DNA insert junctions by size comparison between the secondary and tertiary reaction products.
31. As a result of the nested arrangement of the RB and LB primers within one another (*see* **Fig. 4**), the size profile of the desired PCR product is staggered, decreasing with each TAIL-PCR reaction. The primary reaction typically produces several products. The number of PCR products is reduced with each successive reaction (*see* **Note 30**). In some transformants, more than one PCR product can be

amplified from a single insertion site, presumably because of the presence of multiple annealing sites of AD-1 in the vicinity of the inserted T-DNA.

32. Ectopic transformants expressing the *tk* gene fail to grow on this medium due to their sensitive to F2dU, whereas gene KO mutants are insensitive to F2dU (*see* **Fig. 1**). Add 100 µL of selection medium in each well of 96-well microtiter plate. Alternatively, prepare the medium in Petri plates and spot transformants in a grid format. The concentration of F2dU required for selecting against ectopic transformants varied significantly from one species to another *(27)*. The medium used can also affect sensitivity to F2dU. For a new fungal species, determine the degree of its F2dU sensitivity by comparing the growth of *tk* transformants and untransformed strain on several media amended with varying F2dU concentrations (use 24-well microtiter plate for this testing). Use a concentration that completely blocks the growth of transformants expressing *tk*.
33. Certain fraction of transformants after dual selection appears to be false positive (i.e., ectopic transformants insensitive to F2dU) in both *M. grisea* and *F. oxysporum*. This type of transformants mainly results from truncation of the *tk* gene during T-DNA integration *(27)*. The frequency of false positives varies depending on a number of factors, including fungal species and strains, the target locus, and the length of the gene fragments used for constructing a mutant allele. In fungal species/strains that have a high rate of false positive, PCR prior to single spore isolation (*see* **Note 27**) is highly recommended.

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Green Alga (*Chlamydomonas reinhardtii*)

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Summary

This protocol describes the *Agrobacterium tumefaciens*-mediated nuclear transformation of a microalgae *Chlamydomonas reinhardtii,* using a gene construct carrying the genes coding for β-glucuronidase *(gus)*, green fluorescent protein *(gfp)*, and hygromycin phosphotransferase *(hpt)*. The transformation frequency with this protocol as revealed by hygromycin resistance was many fold higher (about 50-fold) than that of the commonly used glass bead method of transformation. The simplicity of *Agrobacterium*-mediated gene transfer and the high transformation frequency as well as the precision of T-DNA integration will enable further molecular dissection of this important model organism as well as other algal systems to understand basic plant metabolic processes as well as to exploit the systems for biotechnological applications.

Key Words: Algae; *Chlamydomonas reinhardtii*; genetic transformation; reporter genes; *Agrobacterium tumefaciens.*

1. Introduction

A variety of microalgae provide an important source for the production of many valuable compounds and proteins for industrial and pharmaceutical applications (e.g., food additives, polyunsaturated fatty acids, polysaccharides, and hydrogen gas) ***(1)***. In addition, microalgae, particularly *Chlamydomonas reinhardtii,* can be used as powerful model systems for understanding several biological processes in eukaryotic organisms at the molecular level ***(1–5)*** and can also be engineered to act as cell factories to produce several value added proteins, vaccines, antibodies, and anticarcinogenic compounds. However, the exploitation of transgenic microalgae for biotechnological applications is in its infancy because of the difficulties associated with transformation procedures as well as stable expression of heterologous genes ***(1,5)***.

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Different methods have been developed for the nuclear transformation as well as chloroplast transformation of microalgae, especially *C. reinhardtii*. The nuclear transformation was achieved by particle bombardment ***(6–8)***, electroporation ***(9)***, agitation with glass beads ***(10,11)*** or silicon carbide whiskers ***(12,13)***. These transformation protocols, except particle bombardment, utilize cell wall-less strains of *Chlamydomonas* or enzymatic removal of cell wall prior to transformation ***(1)***. Further, these direct gene delivery methods have certain disadvantages, such as the insertion of multiple copies of transgene and a high degree of rearrangements at the site of insertion ***(9,11)***. Therefore, *Agrobacterium*-mediated genetic transformation procedures were developed more recently for *C. reinhardtii* to overcome the above limitations ***(14)*** using a gene construct harboring a fusion of two reporter genes—β-glucuronidase (*gus*), green fluorescent protein (*gfp*), and a selectable marker gene hygromycin phosphotransferase (*hpt*) under the control of a constitutive promoter CaMV 35 ***(5)***.

In brief, this protocol involves the co-cultivation of over-night grown *Agrobacterium* with the thin layer of *Chlamydomonas* cells growing over Tris-acetate phosphate (TAP) agar medium for 2 d in presence of 100 μ*M* acetosyringone (AS). Co-cultivation was followed by the removal of *Agrobacterium* by washing with liquid TAP medium by differential centrifugation and plating over selection medium containing the selection agent hygromycin and bacteriostatic agent cefotaxime. The transformed colonies from the selection medium showed stable integration and expression of the transgenes. A high-transformation frequency (>300 transformants/10^6 cells), which was based on the hygromycin resistance phenotype (i.e., number of hygromycin resistant colonies on the selection medium/total number of cells plated) was achieved, which was about 50-fold higher as compared to the glass bead transformation method.

The *Agrobacterium tumefaciens*-mediated transformation for *C. reinhardtii* described in this chapter may be useful for transforming other algal systems for various purposes, including the understanding of the vital biological processes and biotechnological applications.

2. Materials

2.1. Culture and Maintenance of Chlamydomonas

1. *Chlamydomonas reinhardtii* strain CC-124 (mt^-) (*see* **Note 1**).
2. Tris-acetate phosphate (TAP) medium: Prepare by adding 25 mL/L of the TAP salt stocks, 2.42 g/L Tris, 0.375 mL/L phosfate stock, 1 mL/L glacial acetic acid, and 1 mL/L of Hutner's trace element stock. Adjust pH to 7.0 (*see* **Table 1** for the composition of stock solutions).
3. TAP-agar (TAP medium supplemented with 1.5% w/v agar).
4. Hemocytometer for cell counting.
5. Light microscope.

Table 1
Composition of TAP Medium Stock Solutions

Stock composition	Concentration in stock (g/L)
TAP salt stock (40X)	
NH_4Cl	16.00
$MgSO_4{\cdot}7H_2O$	4.00
$CaCl_2{\cdot}2H_2O$	2.00
Phosphate stock (0.375 mL/L)	
K_2HPO_4	288.00
KH_2PO_4	144.00
Hutner's trace element stock (1000X)*	
Na_2EDTA	50.00
$ZnSO_4{\cdot}7H_2O$	22.00
H_3BO_3	11.40
$MnCl_2{\cdot}4H_2O$	5.06
$FeSO_4{\cdot}7H_2O$	4.99
$CoCl_2{\cdot}6H_2O$	1.61
$CuSO_4{\cdot}5H_2O$	1.57
$(NH_4)_6\ Mo_7O_{24}{\cdot}4H_2O$	1.10

*Components of Hutner's trace element stock were dissolved separately in distilled water. All the components except EDTA were mixed together and boiled and then EDTA was added to this hot mix. pH was adjusted to 6.7 with hot 20% KOH; volume made up to 1 L. The clear green solution was allowed to stand for 2 wk with once daily shaking. The resulting purple solution was filtered through Whatman filter paper, aliquoted, and stored at 4°C. Accurate calculation of element concentration is not possible because of precipitation during preparation. Please refer to Harris *(2)* for more details.

2.2. Transformation

1. Disarmed *A. tumefaciens* strain LBA4404 containing binary vector pCAMBIA1304 carrying hygromycin phosphotransferease (*hpt*) gene under CaMV35S promoter and fused *gfp* and *gus* genes (*gfp:gus*) under CaMV35S promoter (*see* **Fig. 1**) (*see* **Note 2**).
2. TAP medium in 200-mL flasks.
3. Yeast extract mannitol medium (YEM): 0.4 g/L Yeast extract, 10 g/L mannitol, 0.1 g/L NaCl, 0.5 g/L K_2HPO_4, and 0.2 g/L $MgSO_4{\cdot}7H_2O$, pH 7.0. This medium is for raising bacterial cultures to be used in plant transformation experiments (*see* **Note 3**).
4. AS (3,5-Dimethoxy-4-hydrochloro acetophenone) (Aldrich) dissolved in methanol or dimethyl sulfoxide (DMSO) to a concentration of 100 µ*M*.
5. Antibiotics: 50 mg/mL rifampicin stock, 250 mg/mL kanamycin stock, 250 mg/mL streptomycin stock, 50 mg/mL hygromycin B stock, 250 mg/mL cefotaxime stock

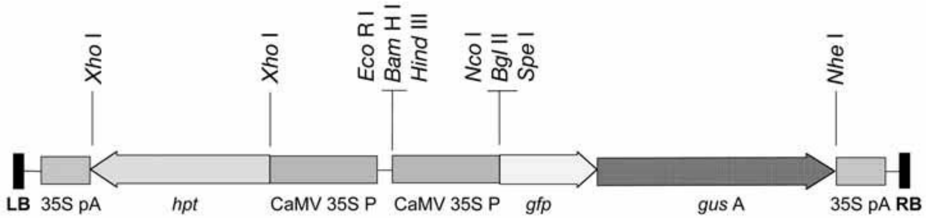

Fig. 1. T-DNA map of pCAMBIA1304. (Reproduced from ***ref.*** ***5*** with permission from Elsevier Science.)

or any other antibiotics depending on the bacterial strains and plant selection marker (*see* **Note 4**).

6. Co-cultivation medium: TAP agar medium containing 100 μ*M* AS in 90-mm Petri plates.
7. Washing medium: TAP liquid medium supplemented with 500 mg/L cefotaxime.
8. Selection medium: TAP agar medium containing 500 mg/L cefotaxime and 10 mg/L hygromycin (*see* **Note 5**).
9. Spectrophotometer.

2.3. Detection of GUS activity

1. X-gluc (5-bromo-4-chloro-3-indolyl-glucuronide) (Sigma).
2. GUS histochemical assay buffer: 10 mg X-gluc dissolved in 2 mL dimethyl formamide, 2 mL 5 m*M* potassium ferricyanide, 2 mL 5 m*M* potassium ferrocyanide. Make volume to 20 mL using 0.1*M* sodium phosphate buffer.
3. Incubator or dry-bath at 37°C.
4. Light microscope.
5. Protein extraction buffer: 50 m*M* Na_2HPO_4, pH 7.0, 10 m*M* β-mecaptoethanol, and 10 m*M* ethylene-diamine tetraacetic acid (EDTA) supplemented with 2 m*M* phenylmethylsulphonyl fluoride (PMSF).
6. MUG (4-methyl umbelliferyl-β-D-glucuronide) (Sigma).
7. GUS fluorimetric assay buffer: Extraction buffer containing 1 m*M* MUG.
8. Stop buffer: 0.2 μ*M* Sodium carbonate.
9. 1 μ*M* 4-methyl umbelliferone (4-MU): Dissolve in stop buffer.
10. Spectrofluorimeter.
11. Others: Standard reagents and materials for protein quantification according to standard procedure ***(15)***.

2.4. GFP Detection

1. Phase-contrast microscope (Nikon Eclipse TE 300 microscope with an excitation filter of 450–490 and a barrier filter at 520 nm).
2. Confocal laser scanning (Radiance 2100, Bio-Rad) using a Nikon microscope (objective Plan Apo 60X/1.4 oil, Nikon, Japan).

2.5. Isolation of Genomic DNA

1. DNA isolation buffer: 2% cetyl trimethyl ammonium bromide (CTAB), 1.4 *M* NaCl, 20 m*M* EDTA, 100 m*M* Tris-HCl, pH 8.0, and 0.2% β-mercaptoethanol.
2. Chloroform:isoamyl alcohol (24:1 v/v).
3. Isopropyl alcohol.
4. Ethanol 70% (v/v).

2.6. Analysis of T-DNA Integration and Transgene Expression

1. PCR Buffer: 10 m*M* Tris-HCl, pH 8.3, 50 m*M* KCl, 2 m*M* $MgCl_2$, 100 μ*M* deoxynucleotide triphosphate (dNTP) mix.
2. Taq-DNA polymerase (MBI, Fermentas).
3. PCR primers: 5′-AGCTGCGCCGATGGTTTCTACAA-3′ (Forward primer) and 5′-ATCGCCTCGCTCCAGTCAATG-3′ (Reverse primer) to amplify 0.5 kb fragment of *hpt* gene.
4. RNeasy plant kit (Qiagen).
5. Protein extraction buffer: 50 m*M* sodium phosfate buffer, pH 7.0, 10 m*M* Na_2EDTA, 2 m*M* β-mercaptoethanol, and 2 m*M* PMSF.
6. Rabbit anti GUS polyclonal antibodies.
7. Goat anti-rabbit IgG—alkaline phosphatase conjugate (Sigma).
8. Others: Materials for Southern and Northern hybridization analysis according to standard procedures (*16*).

2.7. Mating and Genetic Analysis

1. *C. reinhardtii* strain CC-125 (mt^+) (*see* **Note 1**).
2. Gametogenesis media: Low sulfur medium (L-medium), $^1/_5$ strength Nitrogen-free minimal medium (M-N/5 medium) (*see* **Table 2** for the composition of the media).
3. Maturation medium: TAP plus 4% agar.
4. Zygospore germination medium: TAP plus 2% agar.
5. TAP agar plus 10 mg/L hygromycin.
6. Microspatula, blunt glass needles and mouth controlled pipet.
7. Stereo dissection microscope.

3. Methods

3.1. Culture and Maintenance of Chlamydomonas

Unless otherwise mentioned *Chlamydomonas* cultures are grown in liquid TAP medium in an incubator shaker under continuous illumination using cool fluorescent light with 60 μmol/m^2/s at 100 rpm and 23°C. Solid cultures are always maintained on TAP-agar medium. All transformation experiments are initiated from a single colony-derived culture.

3.2. Transformation

1. Inoculate a single colony of *Chlamydomonas* into TAP medium and grow to log phase under illumination.

Table 2
Composition of Media Used for Gametogenesis in *Chlamydomonas*

	Volume of stocks for media (mL/L)	
Stock solution	L- medium	M-N/5 medium
11.4% Na-Citrate·$2H_2O$	5.0	1.0
1% $FeCl_3 \cdot 6H_2O$	1.0	0.2
5.3% $CaCl_2 \cdot 2H_2O$	1.0	0.2
10% $MgSO_4 \cdot 7H_2O$	0.2	0.6
8.34% $MgCl_2 \cdot 6H_2O$	2.8	–
13.1% $K_2HPO_4 \cdot 3H_2O$	1.7	2.4
10% KH_2PO_4	1.0	–
10% NH_4NO_3	3.0	–
16.6% Na Acetate·$3H_2O$	10.0	–
Trace elements*	1.0	0.2
Washed agar	15.0 g	–

*Trace element stock contains 100 mg/L H_3BO_3, 100 mg/L $ZnSO_4 \cdot 7H_2O$, 50 mg/L $MnSO_4 \cdot 7H_2O$, 20 mg/L $CoCl_2 \cdot 6H_2O$, 4 mg/L $CuSO_4 \cdot 5H_2O$, and 15 mg/L ammonium molebdate.

2. Plate about 10^7 cells from the log phase culture on to the solid TAP medium with or without 100 μ*M* AS in 90-mm Petri plates and incubate in light for 2 d to allow a lawn of cell to be formed (*see* **Note 6**).
3. Raise an overnight *Agrobacterium* culture (A_{600} = 0.5) in liquid YEM medium containing appropriate antibiotics (in the present case 10 mg/L rifampicin, 50 mg/L streptomycin, and 50 mg/L kanamycin), pellet and resuspend in liquid TAP medium supplemented with or without 100 μ*M* AS (*see* **Note 7**).
4. Plate 200 μL of the bacterial suspension on to the thin layer of *Chlamydomonas* culture growing on agar plates. Incubate the plates for 2 d at 23°C (co-cultivation) (*see* **Note 8**).
5. Following 2 d of co-cultivation, harvest the cells, wash twice with liquid TAP medium containing 500 mg/L cefotaxime by re-suspending with mild vortexing and centrifugation at 100*g* for 2 min in a microcentrifuge, re-suspend in liquid TAP and plated 2–3 × 10^6 cells on to solid TAP plates containing 10 mg/L hygromycin + 500 mg/L cefotaxime (*see* **Note 9**).
6. Transformed colonies appear in 1 wk (*see* **Note 10**); maintain independent colonies on the selection medium with intermittent sub-cultures.
7. Utilize the transformed colonies maintained in liquid TAP medium under non-selective conditions for molecular analysis (see **Note 11**).

3.3. Detection of GUS Activity

1. For the analysis of *gus* reporter gene expression *(17)* transfer 1 mL of the culture to microcentrifuge tube, pellet the cells by centrifugation at 1500*g* for 5 min and re-suspend in 500 μL of GUS histochemical assay buffer.
2. Incubate cells in the assay buffer at 37°C overnight.

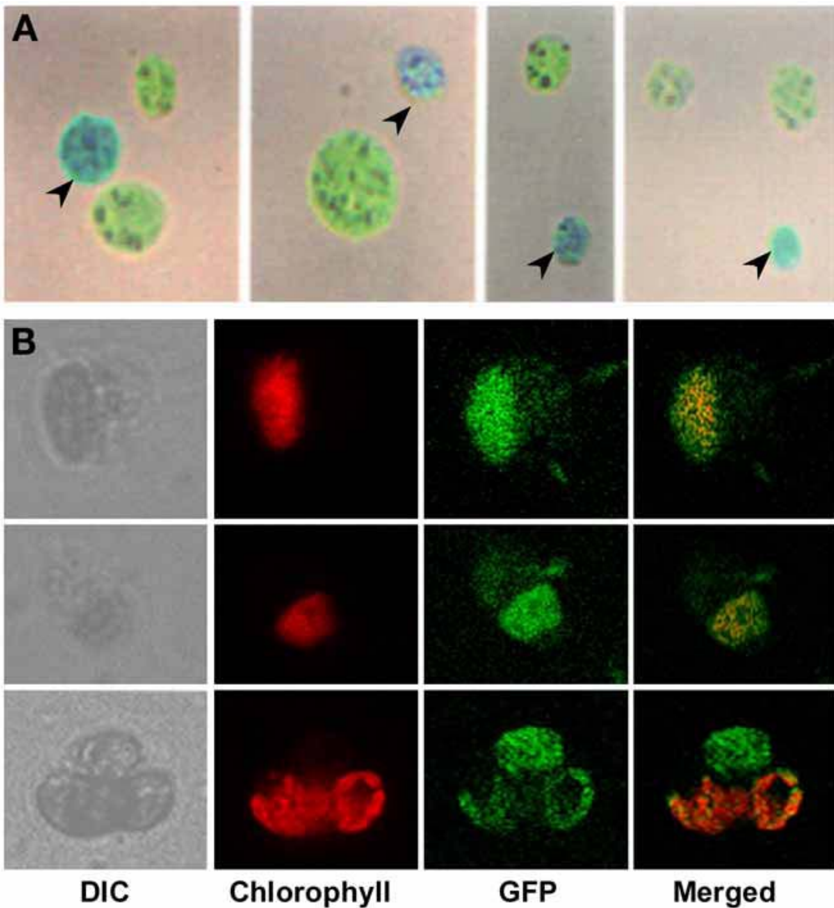

Fig. 2. (**A**) Histochemical detection of GUS activity in the transformed cells of *Chlamydomonas*. Arrow heads indicate the GUS positive cells. (**B**) Detection of GFP in the transformed cells using confocal microscopy. (Reproduced from ***ref. 5*** with permission from Elsevier Science.)

3. Remove the assay buffer by centrifugation, rinse the cells in 70% ethanol, and store in 40% glycerol until used.
4. Observe the cells under light microscope to visualize the GUS expression (*see* **Fig. 2A**).
5. For quantitative detection of the GUS activity, pellet 1 mL of culture in a microcentrifuge at 1500*g* for 5 min at 4°C and homogenize the cells by sonication in the protein extraction buffer.
6. Centrifuge the homogenate at 13000*g* for 15 min at 4°C. Collect the supernatant and use for enzyme assay after quantifying proteins according to standard procedures ***(15)***.
7. Perform GUS assay by adding 100 µL of the extract to 500 µL of pre-warmed assay buffer and incubate at 37°C. Remove 100 µL aliquots at regular intervals and mix with 900 µL stop buffer.

8. Calibrate the fluorimeter or make a calibration curve using triplicate 0 to 100 n*M* 4-MU diluted in stop buffer as for the reaction samples (with excitation at 365 nm and emission at 455 nm) followed by reading the reaction sample fluorescence.
9. Extrapolate the enzyme activity from the standard curve of 4-MU and express as pmoles 4-MU/min/mg protein.

3.4. GFP Detection

1. For detection of green fluorescent protein (GFP) by phase-contrast microscopy, observe the cells mounted on a microscopic slide with a Nikon Eclipse TE 300 microscope with an excitation filter of 450 to 490 and a barrier filter at 520 nm.
2. For confocal microscopy, perform confocal laser scanning (Radiance 2100, Bio-Rad) using a Nikon microscope (objective Plan Apo 60X/1.4 oil, Nikon, Japan). Detect GFP fluorescence with an excitation of 488 nm (argon laser) and high quality band-pass emission filter HQ515/30, centered on 515 nm with 30-nm bandwidth.
3. Detect chlorophyll auto fluorescence through 637-nm red diode. Process the images in Photoshop (Adobe systems) (*see* **Fig. 2B**).

3.5. Isolation of Genomic DNA

1. To isolate genomic DNA from *Chlamydomonas* cells using CTAB buffer ***(18)*** pellet the cultures raised in 100 mL TAP medium to log-phase and re-suspend the cells in pre-heated isolation buffer.
2. Incubate the tubes at 65°C for 1 h with gentle mixing in between.
3. Extract the lysate with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) by gentle mixing and centrifugation at 12,000*g* for 15 min at room temperature.
3. Separate the upper aqueous layer by gentle pipetting into a fresh tube, extract with equal volume of chloroform:isoamyl alcohol and centrifuge as described above.
4. Separate the aqueous layer and mix with two-thirds volume of iso-propyl alcohol in a fresh tube to precipitate the DNA. Mix the contents gently by inverting the tube several times, and centrifuge at 12,000*g* for 15 min at room temperature.
5. Wash the DNA pellet obtained with 70% ethanol, air dry, and dissolve in 100 µL of sterile distilled water.
6. Store DNA samples at −20°C until used for polymerase chain reaction (PCR) or Southern analysis.

3.6. Analysis of T-DNA Integration and Transgene Expression

1. Analyse the transformants by PCR with primers specific to *hpt*. Set up the PCR reaction mixture with 100 ng of DNA from untransformed control as well as putative transgenics with 100 n*M* of the transgene specific primers in 25 µL of PCR buffer containing 0.5 U of Taq DNA polymerase (*see* **Fig. 3A**).
2. For Southern analysis, restrict 10 µg of genomic DNA with *Eco*RI and perform hybridization using ^{32}P-labelled *hpt* gene fragment as a probe using standard procedures ***(16)*** (*see* **Fig. 3B** and **Note 12**).
3. Isolate total RNA from the transformed as well as wild-type cultures using the RNeasy Plant kit (Qiagen) according to manufacturers instructions (*see* **Note 13**).

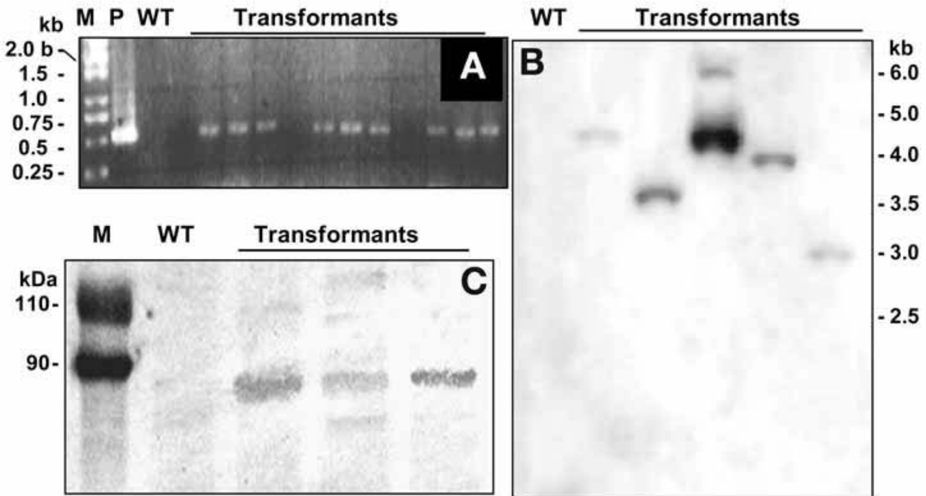

Fig. 3. Molecular analyses of the transformed lines. (**A**) PCR analysis done using *hpt* specific primers. *M*, 1-kb ladder; *P*, pCAMBIA1304 plasmid DNA; *WT*, untransformed control. (**B**) Southern analysis of genomic DNA restricted with *Eco*RI. *WT*, untransformed control. The blots were probed with radiolabelled *hpt* fragment. (**C**) Western analysis for the detection of GFP:GUS protein in the transformed lines. *M*, molecular weight marker; *WT*, untransformed control; and transformed lines [1304]-3, [1304]-7 and [1304]-11. (Reproduced from ***ref. 5*** with permission from Elsevier Science.)

4. Use 30 µg of RNA from the transformed lines and the untransformed control for Northern blot hybridization using ^{32}P labeled *hpt* gene fragment as a probe according standard procedures ***(16)***.
5. Separate soluble proteins (30 µg) extracted from the independently transformed colonies and untransformed cells of *Chlamydomonas* in the extraction buffer by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).
4. After the separation, transfer the proteins on to PVDF membranes by electroblotting. Block the membranes with 3% bovine serum albumin (BSA) for 1 h and incubate with rabbit anti-β-glucuronidase polyclonal antibodies for 1 h and detect by goat anti-rabbit IgG-alkaline phosfatase conjugate (Sigma) using standard procedures ***(16)*** (*see* **Fig. 3C**).

3.7. Mating and Genetic Analysis

1. Prepare cells of both mating types for gametogenesis by growing separately on solid low-sulfur medium (L-medium) for 3 d (*see* **Note 14**).
2. Harvest the nutrient starved cells from **step 1** and resuspended in one-fifth strength nitrogen-free minimal medium (M-N/5) at a density of 2×10^6 cells/mL and incubate with shaking under 18-h light to allow gametogenesis to occur.
3. Harvest gametes by centrifugation at 1500*g* for 5 min and resuspend in fresh M-N/5 liquid medium at a concentration of 0.5×10^8 cells/mL.

4. Mix equal number of both the gametes and allow mating for 1 h. A sample mating mixture can be observed under light microscope to monitor mating. Once mating is complete, plate the mixture onto maturation medium and incubate the plates under light for 24 h followed by incubation in the dark for 6 d to allow for maturation (*see* **Note 14**).
5. Remove the unmated gametes from the maturation plate by gently scrapping with a sterile razor. The zygospores will now be visible clinging on to the agar.
6. Collect the zygospores from the maturation plates using a microspatula or a mouth-controlled pipet under a dissection microscope; place on the germination medium and allow germinating for 1 d under light.
7. The zygospores undergo meiotic division and would be ready for the release of the tetrads in a day. Separate the tetrad products using blunt glass needle and allow producing independent colonies (*see* **Note 15**).
8. Colonies appear in 7 to 10 d. When colonies form, inoculate them into liquid TAP medium and allow to grow to a stationary culture.
9. Spot 5-μL aliquots of the stationary phase cultures on to TAP agar medium, with and without hygromycin, for screening of segregation of hygromycin resistance phenotype.
10. Use the tetrad products for PCR analysis to check whether the hygromycin resistance phenotype and the *hpt* transgene co-segregated (*see* **Fig. 4**).

4. Notes

1. Any other wild-type strain of *Chlamydomonas* may be used. For further details on *Chlamydomonas* strains the readers can refer to Harris *(2)*.
2. Alternatively, any other binary vector suitable for green algae transformation can be used. In all the published works, the major limiting factor for algal transformations has been the promoter driving the transgenes.
3. We found that *Agrobacterium* cultures grown in the minimal medium (YEM) gave better results as compared with a rich medium grown bacterial culture.
4. Antibiotics stocks should be filter-sterilized and added to the autoclaved TAP or YEM medium, which is cooled to about 45°C to get the required final concentration.
5. Hygromycin concentration to be used for selection may vary between systems and it is advisable to do a sensitivity assay to find out the minimum inhibitory concentration for the system concerned.
6. Growing the cells on the plate as a lawn rather than in a liquid medium can be helpful in ensuring the interaction between *Agrobacterium* and the host cell. We have seen that the transformation efficiency was greatly increased on agar culture; whereas the co-cultivation in liquid medium results in low and variable transformation efficiencies.
7. For *Chlamydomonas*, AS is not an essential requirement for transformation events to happen. However, we recommend the use of AS to ensure high-frequency transformation, as the transformation frequency is too low in the absence of AS.
8. *Agrobacterium*-mediated transformation is inhibited at temperatures higher than 28°C, hence, care should be taken while considering the co-cultivation experiment.

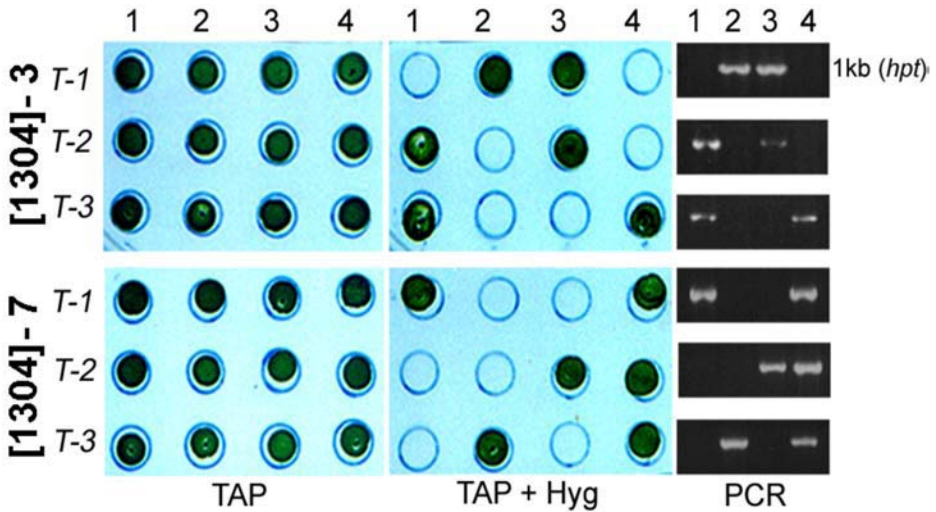

Fig. 4. Genetic analysis of the transformants. Tetrad analysis was performed from crosses between the transformed lines and wild type counter part (mt^+); three complete tetrads were analysed from each of the two transgenic lines [1304]-3 and [1304]-7 tested. The *left panel* shows the growth of the complete tetrads on TAP medium lacking hygromycin. T-1, T-2, and T-3 represent the three complete tetrads from crosses of the respective transformed lines with the wild type CC-125 used for genetic analysis. The *middle panel* shows the growth of the same on TAP + 10 mg/L hygromycin. *Right panel* shows PCR analysis for tetrad progeny using *hpt-* specific primers. (Reproduced from ***ref.*** **5** with permission from Elsevier Science.)

9. *Agrobacterium* cells do not get pelleted at this speed and it will help to wash the *Agrobacterium* cells from the mixture. Although after two or three washes the supernatant will become clear, it is advisable to repeat the washing with mild vortexing till the supernatant becomes clear of bacteria.
10. We get a transformation frequency of 311–355 × 10^{-6} based on the number of hygromycin resistant colonies on selection medium per number of cells plated as compared to the glass bead transformation method (*see* **Table 3**).
11. Although transformed colonies can be maintained on nonselective medium, we recommend that such transformants be maintained on selective medium for a longer period.
12. Choice of restriction enzyme for Southern analysis depends on the vector used. Care should be taken to ensure that the selected enzyme cuts only once within the T-DNA region.
13. While working with RNA take all precautions and use DEPC treated plasticwares in order to prevent the RNA from getting degraded. DEPC is a potential carcinogen and it would be appropriate to wear gloves and protective clothing during the procedure.

Table 3
Transformation Frequencies in *Agrobacterium* and Glass Bead Mediated Transformation of *Chlamydomonas*

Transformation method	Cells plated ($\times 10^6$)	Number of Hyg^r colonies	Transformation frequency (per10^6 cells ± SD)
Agrobacterium			
Co-cultivation on TAP			
	2.10	15	7 ± 1.4
	2.55	20	8 ± 0.8
Co-cultivation on TAP + AS			
	2.65	824	311 ± 10.4
	2.45	869	355 ± 20.2
Glass bead			
	2.30	13	6 ± 1.1
	2.95	24	8 ± 1.3

14. Mating time for strains depends on various factors. In certain difficult to mate strains, it is necessary to have an extended mating period. Care should be taken to plate the mating mixture before the formation of secondary zygospore wall formation.
15. Care should be taken to mark the position of the individual tetrad products. The cells usually cling to the glass needle and this is the most laborious step in tetrad screening.

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Mammalian Cells

Tzvi Tzfira, Talya Kunik, Yedidya Gafni, and Vitaly Citovsky

Summary

Agrobacterium most likely can transform virtually all known plant species, and experimental protocols for *Agrobacterium*-mediated genetic transformation of yet more plant species, ecotypes, and cultivars are published almost on a daily basis. Interestingly, the *Agrobacterium* host range is not limited to the plant kingdom, and it has been shown to transform many species of fungi and even prokaryotes. The ability of *Agrobacterium* to genetically transform HeLa cells further widens the range of potential hosts of *Agrobacterium* to include humans and perhaps other animal species. Furthermore, because mammalian cells significantly differ from plant cells, they provide a useful experimental system for identification and functional characterization of plant-specific factors involved in the transformation process. Here, we present basic procedures for transfection and *Agrobacterium*-mediated genetic transformation of mammalian cells. We also demonstrate the use of mammalian cells for studies of the cellular components of the genetic transformation pathway.

Key Words: Human cells; heterologous transformation system; nuclear import; plant factors.

1. Introduction

Agrobacterium-mediated genetic transformation is the only known natural example of trans-kingdom DNA transfer. In nature, the *Agrobacterium* T-DNA, which carries a set of oncogenic genes, elicits neoplastic growths on the infected plants following its integration into the plant genome ***(1)***, disarmed *Agrobacterium* strains serve, under controlled laboratory conditions, as vectors for introducing recombinant DNA of interest into plant cells both for transient ***(2,3)*** and for stable expression ***(4–7)***. The transformation process requires the presence of two genetic components located on the bacterial tumor inducing (Ti) plasmid: the transferred (T) DNA and the virulence (*vir*) region. The virulence VirD1 and VirD2 proteins are responsible for mobilization of the exported T-DNA copy, a

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single stranded DNA (T-strand) molecule, from the Ti plasmid (*8–12*). The T-strand, together with several Vir proteins, travels into the host cell through a type IV secretion channel formed by the VirB and VirD4 proteins (*13–16*). Inside the host cell cytoplasm, the T-strand likely exists as a DNA-protein transport complex (T-complex) with a single VirD2 molecule attached to its 5′-end and numerous molecules of VirE2, a single stranded DNA binding protein, coating its entire length (*8–10*). Whereas production and export of the *Agrobacterium* T-strand relies exclusively on the function of the Vir proteins, the import of the T-complex into the plant cell nucleus and subsequent uncoating of the T-strand and its integration into the host genome require the active participation of various host cell factors (*8–12*). But are such cellular factors conserved between very different prokaryotic and eukaryotic organisms (e.g., actinomycetes [*17*], yeast [*18–20*], filamentous fungi [*21,22*], cultivated mushrooms [*21*]), and human cells [*23,24*],) all of which can be transformed by *Agrobacterium*? The fact that T-DNA integration in plants occurs by a nonhomologous DNA repair (*25,26*), whereas, in yeast cells it can be directed to both homologous or nonhomologous recombination pathways by different host DNA repair proteins (*27*), suggests that *Agrobacterium* can utilize dissimilar and host-specific cellular pathways for infection. Thus, although some of the basic aspects of the transformation process (e.g., bacterial attachment, and Vir protein, and T-strand export) are likely conserved during transformation of various host species, others (e.g., T-complex nuclear import and T-DNA integration) may occur differently in specific hosts and even under specific physiological conditions of the host cells.

Mammalian cells are fundamentally different from plant cells, and so is their interaction with *Agrobacterium*. For example, plants, unlike mammalian cells, have a cell wall that is rich with phenolic compounds essential for induction of the *Agrobacterium* virulence. Mammalian cells also do not encode a host factor (i.e., the VIP1 protein) essential for the T-complex nuclear import (*28–30*), and human and plant cells differ in their DNA repair systems (*31,32*). Thus, even though *Agrobacterium* is able to transform mammalian cells the process is relatively inefficient, averaging between 10 and 20 stable, antibiotic-resistant transformants/10^6 cells (*23*), which is comparable to the yield of the calcium phosfate technique but lower than that of the lipofectin method (*33*). Future modifications of this system, such as alterations of the bacterial Vir proteins to better conform to the nuclear import machinery of the mammalian cell, are required to position *Agrobacterium*-mediated genetic transformation as a useful tool for the production of transgenic mammalian cell lines. On the other hand, the very same low efficiency with which *Agrobacterium* transforms mammalian cells as compared with plant cells makes mammalian cells a powerful experimental system to study plant-specific aspects of the *Agrobacterium*-host cell interaction. Here we present protocols for *Agrobacterium*-mediated genetic transformation of mammalian cells and

demonstrate how transfected mammalian cells can be used to identify and functional characterize plant-specific factors involved in the transformation process.

2. Materials

2.1. Equipment and Consumables

1. Environmentally controlled shaker (28°C) for *Agrobacterium* culturing.
2. Polymerase chain reaction (PCR) thermocycler for analysis of transgenic cell lines.
3. 37°C incubator with a humidified atmosphere of 5% CO_2/95% air for maintenance of mammalian cell cultures.
4. 80°C dry oven.
5. Disposable 90-mm tissue culture dishes.
6. 90-mm glass Petri dish.
7. 6-well disposable tissue culture plates.
8. 96-well disposable tissue culture plates.
9. 80-mm Whatman filter papers.
10. 22 × 22-mm microscope coverslips (autoclaved) (*see* **Note 1**).
11. Watchmaker forceps (autoclaved).
12. Plate shaker capable of gentle rocking.
13. Inverted and standard light microscopes.
14. Epifluorescence or, preferably, confocal laser scanning microscope.

2.2. Media, Antibiotics, and Chemicals

1. Double-distilled water (ddH_2O); autoclaved.
2. 10X Phosfate buffered saline (PBS) stock solution: 1.37 *M* NaCl, 27 m*M* KCl, 100 m*M* Na_2HPO_4, and 18 m*M* KH_2PO_4, pH 4.7; autoclaved.
3. Dulbecco's modified eagle's medium (DMEM) (GIBCO/BRL).
4. Fetal bovine serum (FBS) (HyClone).
5. DMEM/FBS mixture (90/10 v/v): Made fresh prior to use (*see* **Note 2**).
6. Antibiotic stock solution: Make four 1 mg/mL solutions of each of the following antibiotics—penicillin, streptomycin, spectinomycin, and carbenicillin (*see* **Note 3**).
7. Yeast extract/peptone (YEP) liquid medium: Mix 10 g yeast extract, 10 g Bactopeptone, and 5 g NaCl in 1 L ddH_2O and autoclave.
8. YEP solid medium: Same as YEP liquid medium, only add 15 g/L agar before autoclaving.
9. Acetosyringone (AS) stock solution: Dissolve powdered AS (Sigma-Aldrich) in dimethyl sulfoxide (DMSO) to a stock of 100 m*M*.
10. Cefotaxime stock solution: 2 m*M* dissolved in PBS.
11. Geneticin (G418): 600 µg/mL in H_2O, filter sterilize.
12. Trypsin-ethylene-diamine tetraacetic acid (EDTA) solution: 0.25%.
13. High Pure polymerase chain reaction (PCR) template preparation kit (Roche Molecular Biochemicals).
14. Three (TR1, TR2, and TR3) TAIL PCR-specific nested forward primers.
15. A single degenerate reverse primer (AD2) TAIL PCR primer: 5′-NTCGASTWTS GWGTT-3′, where N is A, C, G T, S is C or G, and W is A or T.

16. Deoxynucleotide triphosfate (dNTP) mix for PCR (0.1 n*M*).
17. TaKaRa EX-Taq polymerase (Pan Vera Corppration).
18. FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals).
19. Paraformaldehyde solution (4% w/v): Mix 4 g in 100 mL PBS, prepare fresh just before use (Fisher).
20. Mammalian permeabilization solution: Mix 500 μL Triton X-100 in 100 mL PBS.
21. 3% Blocking solution: Mix 3 g BSA in 100 mL of 10 m*M* glycine.
22. Antibody solution: Mix 1 g BSA in 100 mL PBS.
23. Mouse and/or rabbit primary antibodies.
24. Fluorescently labeled anti-mouse and/or anti-rabbit secondary antibodies.
25. Mounting antifade medium (Molecular Probes).

2.3. Agrobacterium *Strains, Plasmids, and Mammalian Cell Lines*

1. Human HeLa cell line R19 (*see* **Note 4**).
2. *Agrobacterium tumefaciens* strain C58C1 harboring Ti-plasmid pGV3850.
3. A binary vector (e.g., pNeo) *(23)* carrying in its T-DNA region a neomycin resistance gene expression cassette suitable for selection of transgenic mammalian cells (*see* **Notes 5** and **6**).
4. pEGFP-C1 (Clontech), pcDNA3.1(-)/Myc-His-A (Invitrogen), and pCB6 (34) expression vectors (*see* **Notes 7** and **8**).
5. pEGFP-VirE2 and pEGFP-VirD2 expression constructs (*see* **Note 7**).
6. pcDNA3.1-VirE2-Myc and pCB6-VIP1 expression constructs (*see* **Note 8**).
7. pTA plasmid (Invitrogen) or any other vector for direct cloning of PCR products.

3. Methods

The genetic transformation process begins with induction of the *Agrobacterium vir* gene expression by plant-specific signals, following by bacterial attachment to the host cells. The T-strand and several exported Vir proteins must then travel through the type IV secretion channel into the host cell, through the host cell cytoplasm, and into the nucleus. Within the nucleus, the T-strand is uncoated and is integrated into the host genome, resulting in stable, transgenic expression of the T-DNA encoded genes, one of which should be a selectable marker that allows selection and rescue of the transgenic cell lines. Several key steps of the transformation process can be monitored while establishing a new transformation protocol. These may include *vir* gene induction, bacterial attachment, T-DNA nuclear import, T-DNA transient expression, and T-DNA integration and stable expression. Presented below are general protocols for transformation of a HeLa cell line, monitoring the major steps of the transformation process, and molecular analysis of the resulting transgenic cell lines. We also describe methodology for expression of plant-specific factors in mammalian cells to characterize their role in the transformation process. These protocols can further be modified and expanded to other mammalian cell lines.

3.1. Agrobacterium*-Mediated Genetic Transformation Protocol*

1. Introduce the binary vector with the neomycin resistance gene into *Agrobacterium* using standard $CaCl_2$ transformation methods *(35)*.
2. Two days prior to mammalian cell transformation, prepare a fresh *Agrobacterium* culture by inoculating a fresh colony into 5 mL liquid YEP supplemented with the appropriate antibiotic to maintain the binary vector (e.g., 100 μg/mL spectinomycin for pNeo). Grow in a shaker at 28°C and 250 rpm for 2 d (*see* **Note 9**).
3. One day prior to transformation, place 3 to 5 22 × 22-mm sterile coverslips in a 90-mm tissue culture dish, arranging the coverslips on the bottom of the dish separately from each other. Coverslips that have been autoclaved and stored between Whatman paper filters can be easily transferred to the tissue culture dish using sterile watchmaker forceps.
4. Plate a total of $3–5 \times 10^5$ HeLa cells directly on the coverslips (10^5 cells/coverslip) and culture them in 10 mL DMEM/FBS medium for 1 d at 37°C in the presence of 100 μg/mL penicillin/streptomycin mixture to prevent contamination (*see* **Note 10**).
5. On the day of transformation, replace the HeLa cell culture medium with 10 mL of freshly prepared DMEM/FBS with no antibiotics.
6. Also on the day of transformation, pre-induce *Agrobacterium* by inoculating 5 mL YEP medium supplemented with 100 μg/mL spectinomycin and 100 μ*M* AS with 100 μL of 2-d-old *Agrobacterium* culture from **step 2** (*see* **Note 11**). Grow in a shaker at 28°C and 250 rpm for 4 to 6 h for *vir* gene induction (*see* **Note 12**).
7. For transformation, add 100 μL of the pre-induced bacterial culture from **step 6** (corresponding to between 10^5 and 10^6 bacterial cells) to each tissue culture plate with HeLa cells and incubate for 48 h at 37°C and 5% CO_2 in the presence of 100 μ*M* AS.
8. At different time points of the co-cultivation period (e.g., 2, 8, 12, 24, and 48 h), the attachment of *Agrobacterium* cells to HeLa cells can be examined (*see* **Note 13**). To this end, gently tilt the tissue culture dish, carefully lift a coverslip using a watchmaker forcep, gently blot it dry, place it upside-down on a glass slide, and examine it under a light microscope. An example of a typical *Agrobacterium* attachment to HeLa cells and to petunia protoplasts is shown in **Fig. 1**. The coverslip should be discarded after the observation as it is no longer sterile.
9. Wash the cells twice with 10 mL of pre-warmed PBS (37°C) and culture in 10 mL DMEM/FBS medium for 1 d at 37°C in the presence of 200 μ*M* cefotaxime to kill the bacteria and 600 μg/mL geneticin to select for stably transformed HeLa cells (*see* **Note 14**).
10. Change the selection medium daily and check the plates for bacterial contaminations under a microscope. Once no bacterial cells can be observed, indicating that most Agrobacteria have been eliminated, stop the cefotaxime treatment and continue to **step 11**.
11. Wash the cells twice with 10 mL of pre-warmed PBS (37°C), aspirate the buffer well, and add 2 to 3 mL of trypsin to release the cells. Monitor the cell release under a microscope (*see* **Note 15**).
12. Once the cells are released, re-suspend them in 20 mL DMEM/FBS medium supplemented with 600 μg/mL geneticin by gently pipetting the medium up and down.

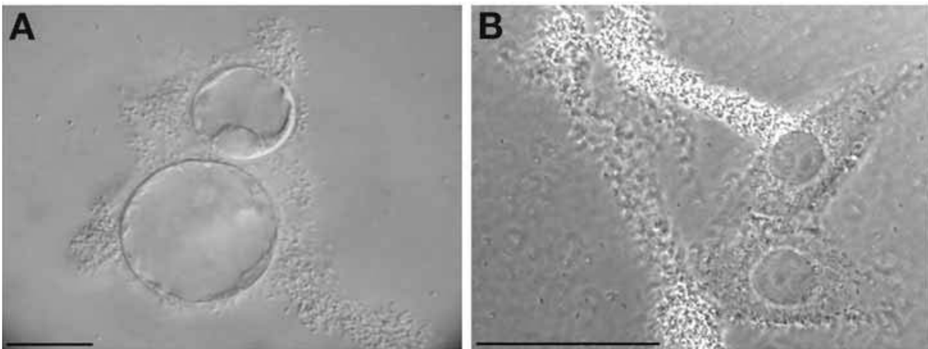

Fig. 1. *Agrobacterium* cells are capable of attaching to cells of evolutionary-distant host species. Similar attachment patterns, with characteristic bacterial aggregates at and around the host cells, were observed when *Agrobacterium* was co-incubated with petunia protoplasts **(A)** and HeLa cells **(B)**. Bars = 50 µm. reproduced from **ref. *23*** with permission from The National Academy of Sciences of the United States of America.

13. Aliquot the cell suspension into a 96-well tissue culture dish (200 µL/well) and incubate at 37°C (*see* **Note 16**).
14. Change the medium daily and monitor for formation of antibiotic-resistant cell foci. These transgenic cell foci should appear 7 to 10 d following step 13 (*see* **Note 17**).

3.2. Analysis of Transgenic Lines Using TAIL PCR Amplification

The ability of HeLa cells stably transformed with neomycin resistance gene to grow in the presence of geneticin is the first indicator for integration of the *Agrobacterium* T-DNA into the host cell genome. Yet to directly demonstrate the T-DNA integration event and to identify the site of integration, thermal asymmetric interlaced (TAIL) PCR is employed to amplify the integration junction for subsequent cloning and sequencing. Before the TAIL-PCR amplification, however, a cell line with a single T-DNA insertion should be identified by Southern blot analysis performed using standard protocols ***(36)***.

1. Using High Pure PCR Template Preparation Kit, extract genomic DNA from a confluent cell culture grown on a 90-mm tissue culture dish and derived from a stably-transformed cell line that carries a single T-DNA insert as determined by Southern blot analysis (see **Fig. 2** and **Note 18** for an example of Southern blot analysis data).
2. Design three T-DNA-specific nested forward primers TR1, TR2, and TR3 located approx 250, 200 and 150-bp, respectively, from the T-DNA right border (*see* **Fig. 3**). Also, prepare a single degenerate reverse primer AD2 (*see* **Note 19**).
3. The T-DNA integration junction is amplified by three consecutive rounds of PCR. Prepare a PCR cocktail with a total volume of 50 µL containing 20 ng DNA (from **step 1**), 0.1 n*M* dNTP, 2.5 m*M* of the TR1 primer, 2.5 m*M* of the AD2 primer, and

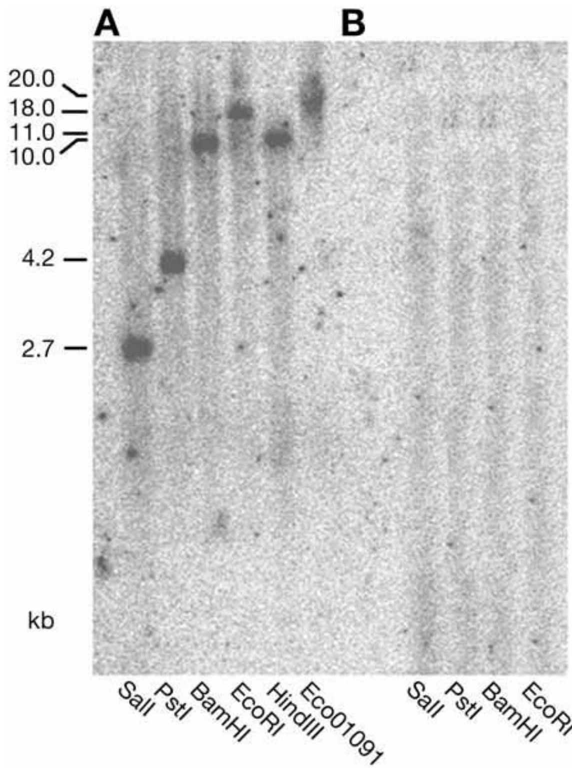

Fig. 2. Southern blot analysis of a transgenic HeLa cell line. Genomic DNA was extracted from cell cultures derived from a cell line stably-transformed with the pNeo plasmid, and purified DNA was digested with the indicated restriction endonucleases. *Pst*I, *Bam*HI, *Eco*RI, and *Hind*III cut once inside the pNeo T-DNA while Eco0109I cuts twice and *Sa*lI does not cut at all. Hybridization with the probe derived from the neomycin resistance gene of pNeo resulted in single bands of various sizes in transgenic **(A)** but not in control, nontransformed HeLa cell lines **(B)**, indicating the presence of a single T-DNA copy within the host cell genome. Reproduced from **ref.** ***23*** with permission from The National Academy of Sciences of the United States of America.

2 units of TaKaRa EX-Taq DNA polymerase with 5 µL of EX-Taq 10X reaction buffer.

4. Set up the first round PCR with the following program: 5 min denaturation at 92°C, 5 cycles of 60 s at 94°C, 60 s at 62°C, and 2.5 min at 72°C, followed by 22 cycles of 30 s at 94°C, 60 s at 68°C, 2.5 min at 72°C, 30 s at 94°C, 60 s at 44°C, and 2.5 min at 72°C (*see* **Note 20**).
5. Dilute the PCR reaction product 1:10, 1:100, and 1:1000 with sterile ddH_2O and subject the diluted samples to a second PCR reaction under the same conditions (*see* **Subheading 3.2.**, **step 3**), but using primer TR2 instead of TR1.

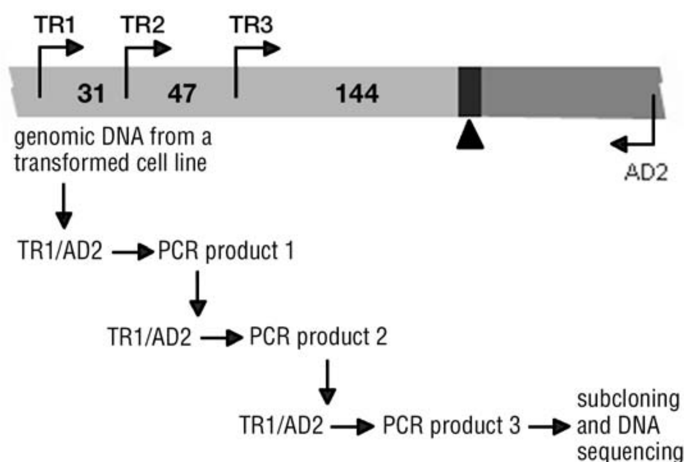

Fig. 3. A basic strategy for TAIL-PCR-based amplification of T-DNA-host DNA integration junctions. Three nested forward primers (TR1, TR2, and TR3), designed to reside within 200- to 300-bp from the T-DNA right border, are used in three consecutive PCR reactions with a degenerate reverse primer (AD2) which anneals within the host DNA. Bar illustrates the amplified junction fragment, and its pNeo T-DNA and HeLa DNA components are indicated by the light-and dark-shaded segments, respectively. The numbers indicate distances between TR1 and TR2, TR2 and TR3, and TR3 and the right T-DNA border. Black box indicates the region of the right T-DNA border, and arrowhead indicates the point of T-DNA integration. Reproduced from **ref. *23*** with permission from the National Academy of Sciences of the United States of America.

6. Dilute products of each of the 3 PCR reactions from **step 5** to 1:10, 1:100, and 1:1000 with sterile ddH_2O and subject the diluted samples to a third PCR reaction under the same conditions (*see* **Subheading 3.2.**, **step 3**), but using primer TR3 instead of TR1.
7. Clone the products of each of the nine PCR reactions—which should represent amplified T-DNA/HeLa DNA junction fragments—from **step 6** into the pTA plasmid (*see* **Note 21**).
8. Sequence your clones and determine the T-DNA/HeLa DNA junction sequence (*see* **Fig. 4** for an example of a T-DNA integration junction in the HeLa cell genome).

3.3. The Use of Mammalian Cells to Study Plant-Specific Components of the Agrobacterium*-Mediated Transformation Process (Transient Transfection of Mammalian Cells)*

Expression of plant-specific factors in mammalian cells provides a very useful and simple system to study the role of these factors in the transformation process, circumventing the need for (not always available) plants with a knockout mutation in the corresponding gene. For example, the effect of VIP1, a plant-

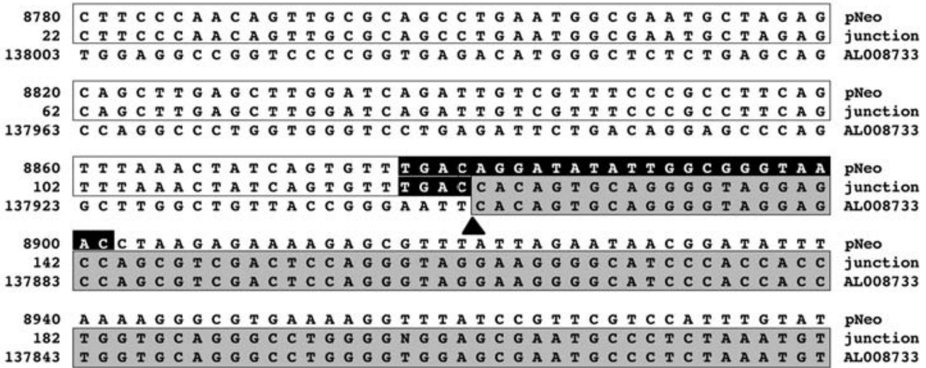

Fig. 4. Nucleotide sequence alignment of the right T-DNA border region of pNeo, the isolated integration junction from an *Agrobacterium*-transformed HeLa cell DNA, and the human genomic DNA Accession No. AL008733. All sequences are shown in the 5′ to 3′ direction. The pNeo sequence is based on the right border region of the parental pPZP221 vector *(37)* (accession no. U10490), and the human DNA sequence is from clone RP1-163G9 (chromosome 1p36.2-36.3). The consensus nopaline-type right T-DNA border sequence *(39,41)* is indicated by a black box. Homology of the junction fragment to pNeo is indicated by open boxes and to the human DNA by shaded boxes. Arrowhead indicts the point of T-DNA integration. Reproduced from **ref.** ***23*** with permission from The National Academy of Sciences of the United States of America.

specific protein that binds VirE2, on VirE2 nuclear import has been discovered and initially characterized in COS-1 and HeLa cells *(28,30)*. In these experiments, a plant factor is transiently co-expressed with an intact or fluorescently-tagged Vir protein, and the intracellular localization of the latter is determined by indirect immunofluorescence or by direct detection of the fluorescent tag. Expression of free or fluorescently-labeled proteins in mammalian cells can be achieved by various transfection methods, one of which is described below.

1. Plate 1–2 × 10^5 mammalian cells directly on a coverslip. Place one coverslip/well in a 6-well tissue culture plate and culture them in 3 mL DMEM/FBS medium/well for 1 d at 37°C in the presence of 100 μg/mL penicillin/streptomycin mixture to prevent contamination (*see* **Note 10**).
2. On the day of transfection, replace the cell culture medium with 3 mL of freshly prepared DMEM/FBS, supplemented with 100 μg/mL penicillin/streptomycin and incubate at 37°C for 3 to 4 h.
3. Prepare 2 to 4 μg of DNA solution (0.5–1 μg/μL) in a sterile 1.5-mL microfuge tube. If a mixture of two plasmids is used, vortex the solution well and centrifuge briefly (2–5 s) (*see* **Note 22**).
4. Place 100 μL of DMEM (without FBS) in a sterile 1.5-mL microfuge tube. Add 3 to 6 μL of the FuGENE reagent directly into the medium and tap it gently to mix.

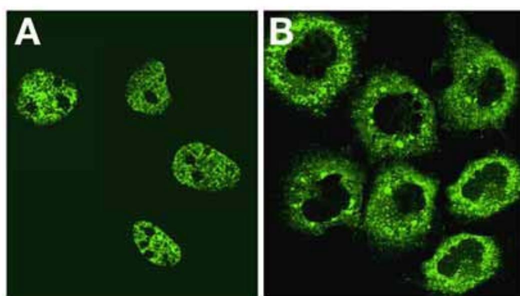

Fig. 5. Expression of GFP-tagged *Agrobacterium* proteins in mammalian cells. The T-strand associated bacterial proteins, known to interact with different host plant factors (*28,42*), differ in their intracellular localization in mammalian cells. While VirD2 efficiently localizes within the mammalian cell nucleus **(A)**, VirE2 is unable to utilize the mammalian nuclear import machinery and remains cytoplasmic **(B)**. All images are single confocal sections. Reproduced from **ref.** *43* with permission from Blackwell Publishing.

5. Add DNA solution from **step 3** to the DMEM-FuGENE mixture. Mix it gently by tapping the tube and incubate at room temperature for 15 to 30 min.
6. Replace the mammalian cell culture medium with 3 mL of DMEM (without FBS) and then slowly add the DMEM-FuGENE-DNA mixture with a pipett, dispersing it evenly over the coverslip.
7. Incubate for 3 h at 37°C.
8. Add 300 µL of FBS directly into the medium and continue incubation at 37°C for 16 to 24 h for optimal expression.
9. For direct visualization of fluorescently-tagged proteins, carefully lift a coverslip using watchmaker forceps, briefly rinse it in PBS, place upside-down on a glass slide, and examine under an epifluorescence or, preferably, under a confocal microscope (*see* **Note 23**). **Figure 5** illustrates the differences in subcellular localization of GFP-tagged VirE2 and VirD2 in COS-1 cells as detected by confocal laser scanning microscopy.
10. For indirect immunofluorescence, aspirate the medium, add 5 mL PBS, and incubate for several seconds to wash the cells; repeat this washing step three times (*see* **Note 24**).
11. Fix the cells for 30 min at room temperature with 5 mL of 3% paraformaldehyde.
12. Aspirate the paraformaldehyde solution, add 5 mL PBS, and incubate for several seconds to wash the cells; repeat this washing step three times.
13. Permeabilize the cells with 5 mL of 0.5% Triton X-100 in PBS for 5 min at room temperature.
14. Aspirate the permeabilization solution, add 5 mL PBS, and wash the cells for 5 min at room temperature by gently rocking the 6-well tissue culture plate; repeat this washing step three times.
15. Block the cells for 1 h at room temperature with 5 mL of 3% (v/w) BSA in 10 m*M* glycine.

16. Aspirate the blocking solution, add 5 mL PBS, and incubate for several seconds to wash the cells; repeat this washing step three times.
17. Dilute the primary antibody in 1% (v/w) BSA in PBS; the extent of dilution depends of the specific antibody titer, but typically 1:100 to 1:1000 dilutions are used.
18. Add 200 to 400 μL of the diluted first antibody to the cells to cover the entire surface of the coverslip. Incubate for 60 min at room temperature.
19. Aspirate the primary antibody solution, add 5 mL PBS, and incubate for several seconds to wash the cells; repeat this washing step three times.
20. Dilute the fluorescently-labeled secondary antibody in 1% (v/w) BSA in PBS; the extent of dilution depends of the specific antibody titer, but typically, 1:100 to 1:250 dilutions are used.
21. Add 200 to 400 μL of the diluted secondary antibody to the cells to cover the entire surface of the coverslip. Incubate for 60 min at room temperature.
22. Aspirate the secondary antibody solution, add 5 mL PBS, and wash the cells for 5 min at room temperature by gently rocking the 6-well tissue culture plate; repeat this washing step three times.
23. Carefully lift the coverslip using watchmaker forceps, briefly rinse it in H_2O, blot it dry while taking care to avoid the direct contact with the cells, mount the coverslip on a glass slide using Antifade mounting medium, and examine the cells under an epifluorescence or, preferably, under a confocal microscope. **Figure 6** illustrates the effect that expression of a plant-specific protein VIP1 has on the subcellular localization of Myc-tagged VirE2 in COS-1 cells as detected using indirect immunofluorescence and confocal laser scanning microscopy.

4. Notes

1. Place several 22 × 22-mm microscope coverslips between 80-mm Whatman paper filters in a 90-mm glass Petri dish. Autoclave for 30 min at 121°C using vacuum dry cycle and dry for several hours in a 80°C oven. Failure to completely oven-dry the coverslips make them sticky and unusable.
2. Pre-warm DMEM/FBS mixture to 37°C before adding the medium to cells to avoid a cold shock.
3. Prepare separate stock solutions for each antibiotic in ddH_2O, filter sterilize and store for up to 30 d at −20°C.
4. Two additional mammalian cell lines have been shown to be transformable by *Agrobacterium*, human embryonic kidney (HEK) 293 and rat clonal pheochromocytoma PC12 neuronal cell lines ***(23)***. Potentially, other mammalian cells lines are also amenable to *Agrobacterium*-mediated transformation.
5. Various *Agrobacterium* binary plasmids can be used. However, most of plant-based vectors contain selection markers under plant-specific promoters. Thus, an expression cassette containing a neomycin resistance (i.e., neomycin phosphotransferase) gene driven by a constitutive promoter active in mammalian cells, such as cytomegalovirus (CMV) promoter or Simian virus 40 (SV40) early promoter, should be cloned into the T-DNA region of the binary vector.
6. The pNeo plasmid employed in the initial studies of *Agrobacterium*-mediated genetic transformation of human cell ***(23)*** contained the neomycin phosphotrans-

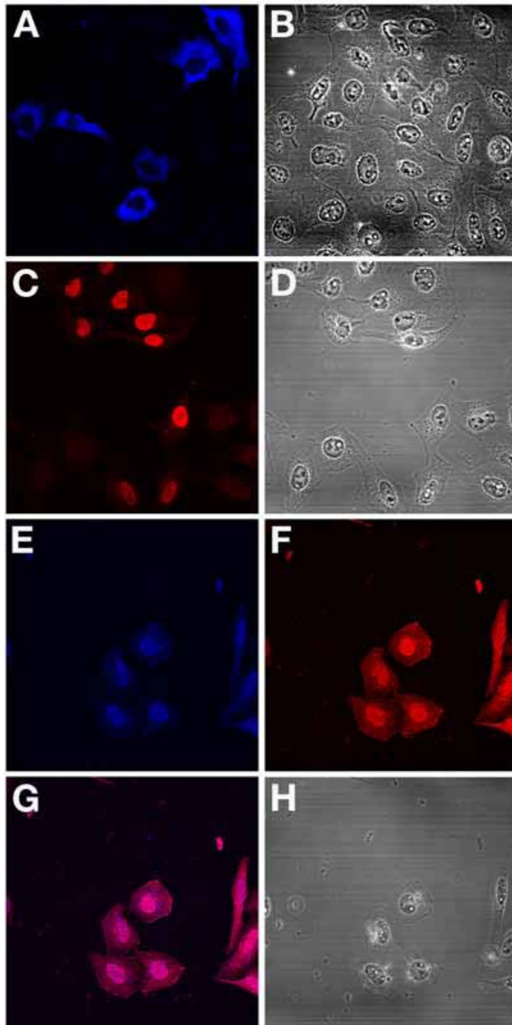

Fig. 6. Indirect immunofluorescence detection of *Agrobacterium* and plant proteins expressed in mammalian cells. Myc-tagged VirE2, similarly to GFP-tagged VirE2 (*see* **Fig. 5**), is not recognized by the nuclear import apparatus of mammalian cells and remains cytoplasmic as detected using primary mouse anti-Myc antibody and secondary Cy5-labeled anti-mouse antibodies **(A**,**B)**. Intact VIP1, a plant-specific protein that binds VirE2 and facilitates its nuclear import in plant cells *(28,29)*, itself efficiently localizes to the mammalian cell nucleus as detected using primary rabbit anti-VIP1 antibodies and secondary Alexa-labeled anti-rabbit antibodies **(C**,**D)**. Myc-tagged VirE2 **(E)** co-expressed with intact VIP **(F)** was redirected into the cell nucleus, resulting in nuclear co-localization of both proteins **(G**,**H)**. *Panels A*, *C*, *E*, *F*, and *G* are fluorescence images; *panels B*, *D*, and *H* are phase contrast images of the corresponding

ferase gene under the control of the SV40 early promoter and Herpes simplex virus thymidine kinase polyadenylation signal (derived from pEGFP-C1, Clontech) cloned into the pPZP221-based binary vector (*37*), between its right and left nopaline-type T-DNA borders.

7. The simplest way to express fluorescently tagged proteins is to clone their encoding gene into the green fluorescent protein (GFP) fusion vector pEGFP-C1 (Clontech) optimized for expression in mammalian cells. The resulting fusion proteins can be directly detected by epifluorescence or confocal microscopy. We used this approach to tag and express *Agrobacterium* VirE2 and VirD2 proteins in HeLa and COS-1 cells.
8. If no antibodies to the target protein are available, it can be labeled with an epitope tag. For example, expression from the pcDNA3.1(-)/Myc-His-A (Invitrogen) vector results in a protein tagged with Myc and/or His epitopes. If the specific antibodies are available, or if the protein is expressed not for detection of subcellular localization, but for studies of its biological activity, its encoding gene can be cloned into a any standard mammalian cell expression vector, such as pCB6 (*34*). Thus produces an intact protein product with no tags. We used this approach to express Myc-tagged VirE2 and intact VIP1 proteins in HeLa and COS-1 cells.
9. Different *Agrobacterium* strains with different Ti and binary plasmids vary in their growth rate. It is important, on the day of infection, to have a freshly and viable *Agrobacterium* cell culture at the logarithmic stage of its growth curve. Thus, several culture transfers or longer growth periods may be required for different *Agrobacterium* strains.
10. Different mammalian cells lines may exhibit different sensitivity to penicillin and streptomycin antibiotics. Other antibiotics or lower antibiotic concentrations should be considered if cells exhibit poor growth at the suggested conditions.
11. Although minimal medium is more conducive to the *vir* gene induction process than the rich YEP medium, rich media are often used to pre-grow *Agrobacterium* during genetic transformation of different plant species (*6,35*).
12. AS is used in many transformation protocols and is essential for induction of the *vir* genes, yet other phenolic compounds may elicit the same inducing effect. Including a control transformation system with uninduced *Agrobacterium* cells can reveal the presence of such compounds that may be either secreted from mammalian cells or represent a component of the growth medium (e.g., pH indicators or serum constituents).
13. *Agrobacterium* infection initiates with attachment of the bacteria to the host cell. Thus, the ability of *Agrobacterium* cells to associate with mammalian cells is an early indicator for a potentially successful transformation process.
14. The choice of antibiotic combination is dictated by the bacterial susceptibility and the selection marker on the T-DNA of the binary vector.

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cells, and *panel G* represents a merged Cy5-Alexa Fluor image. Cy5 is in blue, Alexa Fluor signal is in red, and merged Cy5-Alexa Fluor signals are in pink. All fluorescence images are single confocal sections. Reproduced from **ref. *30*** with permission from the American Society for Biochemistry and Molecular Biology.

15. Trypsin-treated cells will be released from the coverslip into the medium, becoming spherical in shape.
16. Proper dilution will increase the chances of obtaining cell foci derived from a single cell.
17. Transformation conditions are presented for HeLa cells. These conditions were also found suitable for HEK293 and PC12 (*see* **Note 4**), but modification of the transformation protocol may be required for other mammalian cell lines. Furthermore, as described in the **Subheading 1.**, the relatively low efficiency of our transformation protocol suggests that there may still be room for its optimization. Thus, the user is encouraged to vary transformation conditions, such as co-incubation temperatures and growth medium pH, to improve the transformation efficiency.
18. Southern blot analysis is essential to determine the number of T-DNA copies in the genome of the transformed cell line prior to TAIL-PCR, because the efficiency and accuracy of amplification of a specific integration junction site will be greatly reduced if more than one T-DNA copy is present in the genome. Detailed protocols for application of Southern blotting to determine gene copy number are available in nearly every molecular biology protocols book ***(5)***.
19. In plants ***(38–40)***, yeast ***(20)*** and filamentous fungi ***(21)***, integration is more precise and consistent at the T-DNA right border as compared to its left border. Thus TAIL-PCR analysis should first be performed on the right border integration junction. Specific forward primers TR1, TR2, and TR3 should be designed for each T-DNA sequence whereas the degenerate reverse primer AD2 (5′-NTCGASTWTSG WGTT-3′, where N is A, C, G or T, S is C or G, and W is A or T) is expected to anneal within the host cell DNA.
20. After the first round of TAIL-PCR, no integration junction-specific amplification products are expected to be visible on ethidium-stained agarose gels because of the presence of numerous nonspecific DNA fragments. The use of T-DNA-specific nested forward primers in subsequent PCR rounds is expected to substantially enrich the population of the amplified integration junction fragments.
21. Although the final TAIL-PCR round should yield products enriched with the integration junction-specific fragments (*see* **Note 20**), we do not advise gel-purification because of the potential difficulties in their identification among the still-present nonspecific bands. Instead, the entire population of the amplified fragments should be cloned for sequencing analysis. Expect to obtain many nonspecific clones and screen the colonies by digestion with restriction endonucleases specific for the T-DNA region of the binary vector to select the candidate clones for sequencing.
22. Plasmid mixtures at several molar ratios should be prepared and tested when co-transfecting two plasmids. Efficient mixing of DNA solutions is also important to increase the probability of delivering both plasmids into the same cell, thus increasing the proportion of double-transformed cells.
23. Be careful to place the coverslip slowly and directly (squarely) onto the glass slide. Avoid any movement or sliding of the coverslip on the glass slide surface as it may cause severe damage to the cells.

24. All replacements of liquids (i.e., washes, permeabilization, and fixation) should be preformed simply by aspirating the liquid from the well of the tissue culture plate and gently pipetting the new solution into the well.

Acknowledgments

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Mushroom (*Agaricus bisporus*)

C. Peter Romaine and Carl Schlagnhaufer

Summary

We have devised an easy and effective genetic transformation method for the preeminent edible mushroom, *Agaricus bisporus*. Our method exploits the T-DNA transfer mechanism in *Agrobacterium tumefaciens* and relies on the reproductive fruiting body as the recipient tissue. The use of fruiting body explants, particularly the gill, provided high-frequency transformation, overcoming the inefficacy of *Agrobacterium*-based methods targeting fungal spores or vegetative mycelium. The protocol entails incubation of *A. tumefaciens* for 3 h with acetosyringone, a signaling molecule that launches the gene transfer mechanism, co-cultivation of the induced bacterium and gill explants for 3 d, and selection for transformants based on an inherited resistance to the antibiotic hygromycin. Between 7 and 28 d on the selection medium, upwards of 95% of the gill explants generate hygromycin-resistant colonies. About 75% of the mushroom transformants show a single-copy of the hygromycin-resistant gene integrated at random sites in the genome.

Key Words: *Agaricus bisporus*; button mushroom; filamentous fungi; Basidiomycete; *Agrobacterium tumefaciens*; genetic transformation; *Agrobacterium*-mediated transformation; hygromycin.

1. Introduction

A scientific milestone in the field of molecular biotechnology occurred during the 1990s with the discovery that *Agrobacterium tumefaciens*, the bacterial workhorse for gene transfer in plants, could be used to shuttle DNA into fungi (*1,2*). *Agrobacterium*-mediated genetic transformation (Agro-transformation) in fungi was first demonstrated for baker's yeast, *Saccharomyces cerevisiae*, and subsequently extended to numerous filamentous examples ranging from the common *Aspergillus* black mold to the predominant cultivated button mushroom, *Agaricus bisporus*.

Success in experimentally extending the host range of *Agrobacterium* from the plant kingdom to the realm of fungi arose from a detailed knowledge of the

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molecular basis of the gene transfer process in the bacterium-plant interaction *(3)*. Generally speaking, fungi do not produce the phenolic compounds that cue the bacterium to the proximity of a susceptible plant host. However, by simply providing an exogenous source of a signaling phenolic compound, such as acetosyringone (AS), the virulence genes of *A. tumefaciens* are activated in the normal fashion, leading to the cascade of events culminating in the transfer of DNA into the fungal genome. In essence, *A. tumefaciens* can be lured into sensing a non-host fungus as a susceptible plant.

Today, Agro-transformation is proving highly effective for a rapidly growing number of fungal species, many of which have resisted traditional methods for gene transfer (i.e., direct uptake by protoplasts and biolistics). Moreover, the inter-kingdom delivery of genes by *Agrobacterium* is not limited to fungi, because the mechanism was shown to operate with HeLa cells *(4)*. Thus, Agro-transformation methodology will likely be applicable to most, if not all, fungi.

The original Agro-transformation method reported by de Groot et al. *(2)* proved too inefficient to be applied to the breeding and scientific investigation of *A. bisporus*. Shortly thereafter, we described a facile *Agrobacterium*-based protocol that provided high-frequency transformation *(5)*. In our investigations, the tissues comprising the fruiting body (= mushroom), particularly the gill, were found to be highly receptive to DNA transfer mediated by *A. tumefaciens*. The use of gill explants overcame the low efficiency and poor reproducibility observed with the use of the spores or vegetative mycelium *(2,6–8)*.

de Groot et al. *(2)* used spores as the recipient tissue and obtained a 0.00003% transformation efficiency for *A. bisporus*, while Chen et al. *(5)*, relying on gill explants, achieved rates of 30 to 40% (e.g., 30% = 30 of 100 tissue explants generating hygromycin-resistant colonies). Today, we routinely achieve rates of between 60 and 95%. However, our efficiencies are not directly comparable to those of de Groot and coworkers, because the unit of transformation differs (i.e., spore vs cell cluster). For example, simply increasing the size of the explant (more target cells) increases the apparent efficiency. Nonetheless, our method offers a high "experimental efficiency" or perhaps "effectiveness" is a more appropriate term. Whichever, acquiring large populations of *Agaricus* transformants for research or breeding purposes can now be achieved with ease. Also, our method has proven sufficiently robust to be reproduced by other researchers *(7,9–11)*.

2. Materials

2.1. Agaricus *Fruiting Bodies and* Agrobacterium *Strain/Vector*

1. White button mushrooms: Widely available in food markets (*see* **Note 1**).
2. *Agrobacterium* vector pBHg *(5)*: A physical map highlighting the key features of this vector appears in **Fig. 1**. (available from C. Peter Romaine, Penn State, University Park, PA) (*see* **Note 2**).

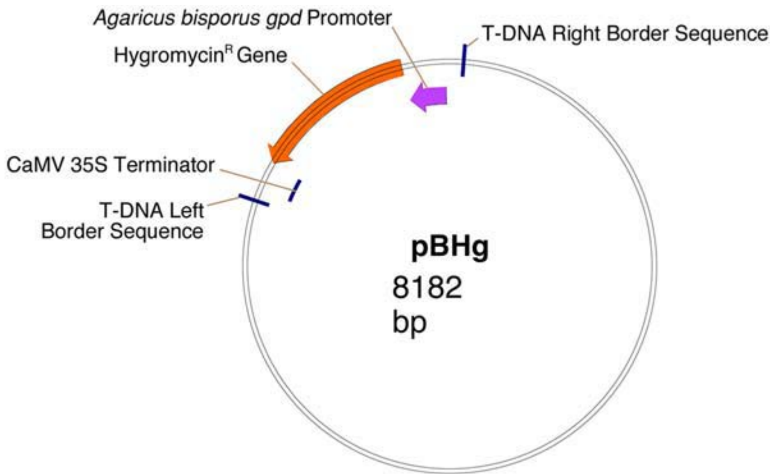

Fig. 1. Organization of binary plasmid vector pBHg. Shown are the hygromycin B resistance gene, *Agaricus bisporus* glyceraldehyde 3-phosphate dehydrogenase (*gpd*) promoter, *Cauliflower mosaic virus* (CaMV) 35S terminator sequence, and *Agrobacterium tumefaciens* T-DNA border sequences.

3. *Agrobacterium* strain AGL-1: Available from Mark Guiltinan (Penn State, University Park, PA) (*see* **Note 3**).

2.2. Stock Solutions

1. 50 mg/mL Kanamycin solution: 500 mg of kanamycin in 10 mL of water. Using a 0.2-µm syringe filter, filter-sterilize the solution. Store at –20°C.
2. K7 buffer: 100 g of dibasic potassium phosphate (K_2HPO_4) and 72.5 g of monobasic potassium phosphate (KH_2PO_4) in 500 mL of water. The pH should be 7.0.
3. M-N solution: 15 g of magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) and 7.5 g of NaCl in 500 mL of water.
4. 1% $CaCl_2$ solution: 1 g of calcium chloride dihydrate in 100 mL of water.
5. 20% Glucose solution: 20 g of glucose in 100 mL of water.
6. 0.01% $FeSO_4$ solution: 0.01 g of ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) in 100 mL of water.
7. Element solution: 50 mg of each of the following: zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), cupric sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), boric acid (H_3BO_3), (manganese sulfate monohydrate) ($MnSO_4 \cdot H_2O$), and disodium molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$) in 500 mL of water.
8. NH_4NO_3 solution (20%): 20 g of ammonium nitrate (NH_4NO_3) in 100 mL of water.
9. K4 buffer: 109 g of K_2HPO_4 in 500 mL of water. Adjust to pH 4.9 with phosphoric acid. This buffer might precipitate upon storage. If so, warm the solution until it appears clear.
10. Glycerol solution (50%): 50 mL of glycerol and 50 mL of water.

11. 1 *M* MES buffer: 106.62 g of MES monohydrate ($C_6H_{13}NO_4S.H_2O$) in 500 mL of water. Adjust to pH 5.5 with NaOH. This buffer might precipitate upon storage. If so, warm the solution until it appears clear.
12. 100 m*M* Acetosyringone (AS) solution: Prepare just before use. 0.197 g of AS (Sigma-Aldrich, St. Louis, MO) in 4 mL of 70% ethanol solution. Filter-sterilize.
13. Hygromycin solution (30 mg/mL): 30 mg of hygromycin B in 1 mL of water. Filter-sterilize and store at –20°C.
14. 200 m*M* Cefotaxime solution: 0.10 g of cefotaxime (sodium salt) in 1 mL of water. Filter-sterilize and store at –20°C.
15. 100 mg/mL Moxalactam solution: 100 mg of moxalactam (sodium salt) in 1 mL of water. Filter-sterilize and store at –20°C.
16. 12.5 mg/mL Tetracycline solution: 125 mg in 100 mL of 70% ethanol. Store at –20°C.
17. Sterile water: Autoclave 2 L of water for 20 min.
18. Bleach solution (10%): 100 mL of commercial bleach solution and 900 mL of water. Prepare fresh for use.
19. LETS buffer: 0.42 g of lithium chloride (LiCl), 0.37 g of ethylene-diamine tetraacetic acid (EDTA), 0.24 g of Tris Base, and 0.5 g of sodium diodecyl sulfate (SDS) in 100 mL of sterile water. Adjust to pH 7.8 with HCl and store at 4°C.
20. Phenol/chloroform/isoamyl alcohol mixture: Equal volumes of water-saturated phenol (available from commercial sources) and a chloroform/isoamyl alcohol mixture (24/1 [v/v]). Store at 4°C.
21. Ethanol solution (70%): 70 mL of 95% ethanol and 25 mL of sterile water. Store at –20°C.

2.3. Culture Media

As a rule, prepare the four transformation media (minimal, induction, co-cultivation, and selection) fresh for each experiment from filter-sterilized stock solutions stored at 4°C. The only exception to this rule is the 100 m*M* AS solution, which is prepared just before use from the crystalline form stored at room temperature (RT) (*see* **Note 4**). Prior to storage, filter-sterilize all solutions using a 0.2-µm syringe filter or filter system, which are available from commercial sources.

1. Luria–Bertani (LB) medium: 10 g of bacto-tryptone (Difco, Detroit, MI), 5 g of yeast extract (Difco), and 5 g of NaCl in 1 L of water. Adjust to pH 7.0 with 10 *N* NaOH. Dispense 100-mL portions into 250-mL glass bottles. For solid medium, add 15 g of agar. Autoclave for 20 min and store at room temperature (RT).
2. LB agar + kan (50 µg/mL): Prepare 1 L of LB agar, allow it to cool to about 50°C, and then add 1 mL of kanamycin solution. Mix the solution by gently swirling the flask. Dispense 30-mL portions into 9-cm Petri plates. Store the plates in sealed plastic bags at 4°C.
3. Minimal medium (MM): 5 mL of K7 buffer, 10 mL of M-N solution, 0.5 mL of $CaCl_2$ solution, 5 mL of glucose solution, 5 mL of $FeSO_4$ solution, 2.5 mL of

element solution, 1.25 mL of NH_4NO_3 solution, 2 mL of kanamycin solution, and 468.75 mL of sterile water (*see* **Note 5**). For each transformation of *A. bisporus* to be carried out, dispense a 100-mL portion into a 250-mL sterile flask.

4. Induction medium (IM): 0.4 mL of K4 buffer, 10 mL of M-N solution, 0.5 mL of $CaCl_2$ solution, 2.5 mL of element solution, 1.25 mL of NH_4NO_3 solution, 5 mL of glycerol solution, 20 mL of MES buffer, 5 mL of glucose solution, 2 mL of kanamycin solution, 2 mL of AS solution, and 451.35 mL of sterile water (*see* **Note 5**).
5. Co-cultivation medium (CCM): prepare IM, but with 2.5 mL of glucose solution (i.e., $^1/_2$ rate) and 7.5 g of agar (*see* **Note 6**). Dispense 30-mL portions into 9-cm Petri plates. Once the medium has solidified, overlay a sterile filter paper circle on the medium in each plate, being careful not to trap air bubbles beneath the paper.
6. Selection medium (SM): 10 g of malt extract (Difco), 1.05 g of MOPS (4-morpholinepropanesulfonic acid), and 7.5 g of agar in 500 mL of water. Adjust to pH 7.0 with KOH. Autoclave for 20 min. When the medium reaches about 50°C, add 0.5 mL of each of the antibiotic solutions, hygromycin, cefotaxime, and moxalactam. Swirl the flask to mix the solution. Dispense 30-mL portions into 9-cm Petri plates.
7. Malt agar (MA): 2 g of malt extract, 2.1 g of MOPS, and 15 g of agar in 1 L of water. Adjust to pH 7.0 with KOH. Autoclave for 20 min. When the medium reaches about 50°C, add 4 mL of tetracycline solution. Swirl the flask to mix the solution. Dispense 10-mL portions into 5.5-cm Petri plates. Store the plates in sealed plastic bags at 4°C.

2.4. Other Materials

1. Filter paper circles: 7-cm dia. quantitative filter paper circles (Grade No. 42, Whatman). Autoclave for 20 min.
2. 250-mL Side-arm flask: Plug the side arm with cotton and insert a rubber stopper in the mouth. Wrap the entire assembly with aluminum foil. Autoclave for 20 min.
3. Rye grain: Premium quality (Hesco Inc., Watertown, SD 57201).
4. 500-mL Erlenmeyer flasks and cotton plugs.
5. Calcium carbonate ($CaCO_3$).
6. Casing mixture: On a volume basis, mix two parts peat humus (Conrad Fafard, Inc., Agawam, MA) to one part agricultural lime (Marcal Kao-Clean Absorbent, Marcal Paper Mills, Inc, Elwood Park, NJ).
7. 32 oz Plastic containers: Widely available in the deli section of food markets.

3. Methods

Getting started: If you have AGL-1 cells to be transformed with the vector pBHg, then proceed to **Chapter 3**, Volume 1. If you have cells already carrying pBHg, then proceed to **Subheading 3.1.**

3.1. Preparation of Agrobacterium Culture

1. Three days before transformation experiment, streak the AGL-1 cells, which were confirmed positive by polymerase chain reaction (PCR) analysis for carrying

pBHg and had been stored at −80°C, onto a plate of LB agar + kan. Grow the culture for 2 d at 28°C.

2. In the morning of the day before transformation experiment, transfer a single colony of AGL-1 from the LB agar + kan to 100 mL of MM. Grow the culture overnight at 28°C with shaking at 250 rpm (*see* **Note 7**).
3. The day of transformation, when the overnight culture has grown to an OD_{600} of 0.6 to 0.8 (generally mid to late morning), collect the cells by centrifugation at 3000*g* for 15 min.
4. Resuspend the cells in 100 mL of IM by gently swirling the tube. Incubate the bacterial suspension for 3 h at room temperature with shaking at 100 rpm.

3.2. *Transformation of* Agaricus bisporus

3.2.1. *Preparation of Gill Explants*

1. About 15 min before the completion of the 3-h induction period for the AGL-1 cells, surface-sterilize fruiting bodies with unexposed gills by soaking with occasional agitation in 500 mL of bleach solution for 1 min (*see* **Note 8**).
2. Decant the bleach solution and rinse the fruiting bodies by soaking with occasional agitation in 500 mL of sterile water. After about 20 s, decant the water and repeat the water rinse twice.
3. Using a sterile scalpel blade, excise the veils from the fruiting bodies. Harvest the exposed gill tissue and section it into 2 to 5-mm pieces.

3.2.2. *Co-cultivation of* Agrobacterium *and* Agaricus

1. Following the 3-h induction period, transfer the AGL-1 cells from the shaker flask to a sterile 250-mL side-arm flask.
2. Transfer upwards of 300 gill explants to the side-arm flask with the AGL-1 cells. Seal the mouth of flask with a rubber stopper.
3. Apply a vacuum (Edwards 8 Two Stage High Vacuum Pump, Sussex, England) to the flask for 7 to 10 min or until emanation of air bubbles from the gill tissue has ceased. When the tissue pieces have been thoroughly purged of air, they will settle to the bottom of the flask.
4. Decant the *Agrobacterium* suspension and transfer upwards of 200 gill explants to a plate of CCM. Distribute the explants uniformly in order to maximize their surface contact with the filter paper overlaid on the medium.
5. Incubate the plates of CCM for 3 d at 20 to 22°C in an opaque storage box (i.e., in the dark) (*see* **Note 9**).

3.2.3. *Selection of* Agaricus *Transformants*

1. Following the 3-d co-cultivation period, transfer 25 gill explants to each plate of SM, spacing the explants equidistantly at about 1-cm intervals. Incubate the plates in the dark at room temperature (*see* **Note 10**).
2. After 7 d, and for the next 3 wk, observe the plates daily for the appearance of mycelial strands radiating outward from the margins of the gill explants (*see* **Note 11**).

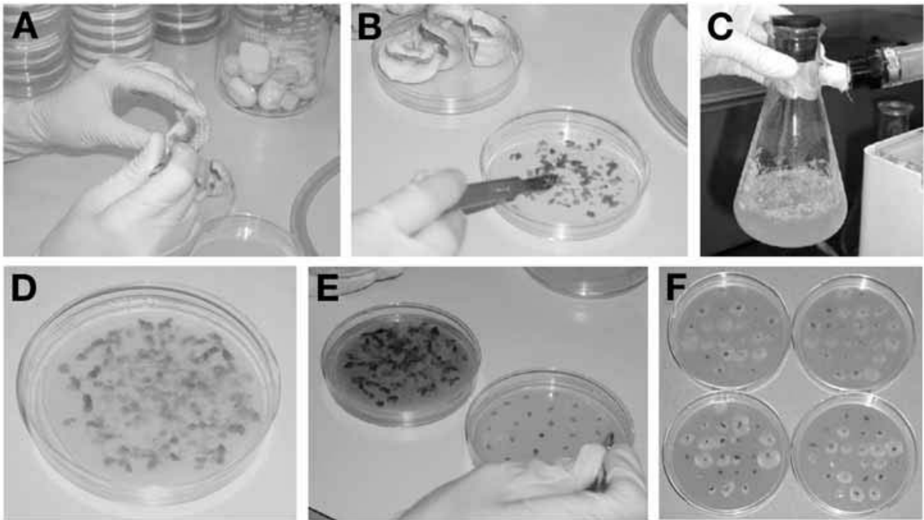

Fig. 2. Fruiting body Agro-transformation method for *Agaricus bisporus*. **(A)** Gill tissue is harvested from mushrooms with intact veils, **(B)** sectioned into 2- to 5-mm pieces, **(C)** vacuum infiltrated with acetosyringone-induced *Agrobacterium tumefaciens* AGL-1 carrying vector pBHg, which contains the *hph* gene conferring hygromycin resistance, **(D)** co-cultivated with AGL-1 for 3 d, and **(E)** plated on selection medium containing hygromycin. **(F)** Selection plates after 2 wk showing high-frequency regeneration of putative hygromycin-resistant transformants.

Figure 2 depicts the co-cultivation and selection steps in the fruiting body tissue protocol. **Fig. 2F** shows an example of the appearance of SM plates after 2 wk. **Figure 3** provides an example of the time-course for the appearance of putative hygromycin-resistant transformants on SM.

3. Once a transformant has grown into a plainly visible colony, transfer it to an individual plate of MA (*see* **Note 12**).
4. Maintain transformants on plates of MA at RT, transferring the cultures at 4- to 6-wk intervals to fresh plates of MA.

3.3. Extraction of Agaricus *DNA for PCR and Southern Analyses*

1. After about 3 wk of growth on the MA, scrape mycelium from an approximate 1-cm dia. area of a transformant culture, being careful to avoid as much agar as possible. Transfer the mycelium to a 1.5-mL microfuge tube (*see* **Note 13**).
2. Add 400 µL of LETS buffer to the tube. Using a disposable micropestle and a hand-held motorized driver, thoroughly homogenize the mixture.
3. Add 500 µL of phenolichloroformiisoamyl alcohol mixture to the tube and emulsify the homogenate by vortex mixing for 30 s.
4. Centrifuge the tube at 16,000*g* for 3 min, and transfer about 300 µL of the upper aqueous phase to a clean microfuge tube.

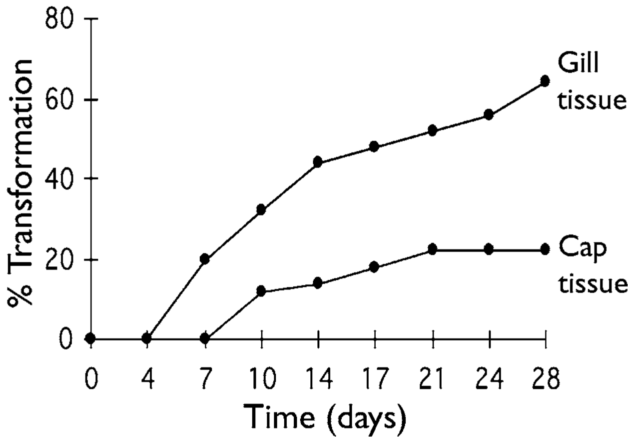

Fig. 3. Time-course for the appearance of hygromycin-resistant transformants from gill and cap explants of fruiting bodies. Selection medium contained 30 µg/mL hygromycin.

5. Add 600 µL of ice-cold 95% ethanol and invert the tube several times rapidly to mix the solution.
6. Centrifuge the tube at 16,000*g* for 5 min and decant the alcohol. Wash the pellet with 500 µL of ice-cold ethanol solution by vortex mixing for 10 s.
7. Centrifuge the tube at 16,000*g* for 5 min and decant the alcohol. Air dry the pellet and resuspend it in 50 µL of sterile water.
8. DNA is ready for PCR and Southern analysis. Our typical yield is about 80 µg of DNA (*see* **Note 14**).

3.4. Fruiting of Transformant Cultures

1. For each transformant culture to be fruited, add 100-mL of rye grain, 40 g of calcium carbonate, and 100 mL of warm tap water to a 500-mL Erlenmeyer flask. Seal the flask with a cotton plug and autoclaved for 45 min at 121°C (fast exhaust mode).
2. When the grain has cooled to the touch, transfer 2 to 3 mycelial agar pieces (about 1 cm each) from a transformant culture to the flask. Shake the flask to evenly distribute the mycelial inoculum.
3. Incubate the grain flasks for 3 to 4 wk at room temperature or until the grain has become thoroughly colonized by the mycelium of the mushroom fungus. Grain flasks should be shaken at weekly intervals to redistribute the mycelium and thereby hasten colonization.
4. Transfer the mushroom fungus-colonized grain (= spawn) from the flask to a plastic container.
5. Immediately overlay the spawn with a 2.5-cm depth of casing mixture, and incubate the containers at room temperature. *Agaricus bisporus* can be fruited in the dark, but light will not impede the process.

6. Water the casing mixture daily such that the moisture level of the peat reaches field capacity after 2 to 3 d. Maintain the casing at field capacity throughout the cropping cycle.
7. When about 50% of the surface area of the casing has become colonized by the mushroom mycelium (about 1 wk), reduce the temperature to 18°C in order to induce fructification. Maintain this temperature throughout the fruiting cycle.
8. Harvest fruiting bodies as they develop and mature. Typically, the first ones will appear at about 2 wk after the temperature induction and others will continue to emerge at weekly intervals for approximately one month.

4. Notes

1. All white button mushrooms grown throughout the Americas and Europe represent a single genotype referred to as the 'U1' hybrid off-white strain. Therefore, any commercial source of white fruiting bodies can be used as the recipient tissue for transformation experiments. Also, the brown variety of *A. bisporus* sold under the name "portabello" is a suitable source of explants. We have had no experience, however, with other genotypes of *A. bisporus* that are grown throughout Asia.
2. Vector pBGgHg was used our original description of the procedure *(5)*. pBHg is identical to this vector except it lacks the green fluorescent protein (*gfp*) gene. Otherwise, both consist of a pCAMBIA 1300 (CAMBIA, Canberra, Australia) backbone with the *Escherichia coli* hygromycin B phosphotransferase (*hph*) gene controlled by the *A. bisporus* glyceraldehyde 3-phosphate dehydrogenase (*gpd*) promoter and *Cauliflower mosaic virus* 35S terminator, all of which are flanked by the *Agrobacterium* T-DNA border sequences. Any DNA sequence placed between these border sequences will become integrated into the *A. bisporus* genome. Hygromycin resistance is used as a selectable marker for identifying transformants in *A. bisporus*. Within the pCAMBIA sequence is situated the gene conferring kanamycin resistance, which is used as a selectable marker for maintenance of the plasmid in *Agrobacterium*. Plasmid DNA can be stored in aliquots in TE buffer at −80°C.
3. We also have used *Agrobacterium* strain EHA-105 with good success. To make a stock culture mix equal volumes of an overnight broth culture and a 10% glycerol solution. Store in aliquots at −80°C.
4. AS is a phenolic compound that loses its potency upon oxidation. For each experiment, we prepare a fresh AS solution from the crystals stored at room temperature, rather than from a stored stock solution in ethanol. Also, we are keen to replenish the AS crystals when they develop a noticeably brown color. When troubleshooting low-transformation efficiencies the AS should be considered.
5. Pre-mix the various buffer and salt solutions. Autoclave the water with a magnetic stir bar in a 1-L flask for 20 min. Once the water has cooled to about 50°C, add the pre-mixed solution, and then thoroughly stir the complete mixture.
6. Pre-mix the various buffer and salt solutions. Autoclave the water and agar with a magnetic stir bar in a 1-L flask for 20 min. Once the agar solution has cooled to about 50°C, added the pre-mixed solution, and then thoroughly stir the complete mixture.

7. MM, as its name implies, is not particular rich in nutrients. For this reason, we always start our overnight culture with an entire colony of cells sampled early in the morning, just to ensure that the cells have grown sufficiently by mid to late morning of the following day.
8. Several-hundred gill explants can be harvested from a single average-size fruiting body. Appreciably high rates of transformation also can be obtained using the spongy tissue comprising the cap and stem of the fruiting body (*see* **Fig. 3**).
9. In our experience, it is important to maintain a temperature of 20 to 22°C during the co-cultivation step in order to achieve the maximal transformation efficiency. We have explored temperatures ranging from 18 to 28°C and observed that transformation ensues over a fairly broad range (18 to 26°C), but with 28°C being highly inhibitory and with a trend towards higher rates at the lower temperatures. Troubleshooting low-transformation efficiencies should consider the temperature during co-cultivation.
10. At this spacing, there is ample room for transformants to regenerate sizeable colonies that can be easily subcultured before coalescing with neighboring colonies.
11. As early as 7 d and as late as 9 d after plating the explants on SM, it should be possible to observe the first appearance of hyromycin-resistant transformant colonies. Using a magnifying glass with the aid of backlighting, fine mycelial threads can be seen extending outward into the medium, often beginning at the angular corners on the explants. We allow the colonies to grow for about 1 wk on SM before subculturing to MA. Generally speaking, the earlier the transformants are first observed on the selection plates, the higher the overall transformation rate for a given experiment will be (i.e., 60 vs 95%).
12. Any questionable transformants growing on SM containing 30 µg/mL of hygromycin can be transferred to selection plates containing 50 µg/mL of the antibiotic for confirmation. For all practical purposes, *A. bisporus* cultures capable of growing at this higher rate are bona fide transformants.
13. Alternatively, putative transformants can be grown at room temperature as stationary cultures in 50 mL of MA broth (MA without agar). After several weeks of growth, harvest the mycelium by passing it through a piece of qualitative filter paper and use 100 mg for DNA extraction.
14. While the LETS procedure yields DNA that is a suitable template for the PCR, on occasion we have experienced difficulty in cutting the DNA with some restriction enzymes. Therefore, we often use the cetyltrimethy ammonium bromide (CTAB) procedure ***(12)*** for Southern analysis, which generates a higher purity DNA template but at somewhat lower yield and lesser convenience than the LETS method.

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Yeast (*Saccharomyces cerevisiae*)

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Summary

The yeast *Saccharomyces cerevisiae* is one of the best characterized eukaryotic organisms. This species has enabled a detailed study of the (genetic) requirements for *Agrobacterium*-mediated DNA transformation. For instance research with this yeast has led to the recognition that the transforming DNA molecules integrate into the eukaryotic chromosomes either by homologous recombination, which is the preferred pathway in *S. cerevisiae*, or by nonhomologous end-joining. Based on the protocol for *Agrobacterium*-mediated transformation of *S. cerevisiae* methodology has been developed for the transformation of many other yeast and fungal species.

Key Words: Yeast; binary vector; T-DNA integration; gene targeting; gene tagging.

1. Introduction

Agrobacterium tumefaciens is a Gram-negative soil bacterium which is in use as a versatile genetic engineer of plants ***(1)***. This bacterial vector introduces a nucleoprotein complex consisting of a single stranded (ss) DNA molecule (T-strand) with a 5′ covalently attached VirD2 protein into the recipient plant cells ***(2)***. The VirE2 protein, a ssDNA binding protein, is separately introduced into the recipient cells, where it cooperatively binds to the T-strand ***(3)***. The VirD2 and VirE2 proteins protect the T-strand against nucleases, prevent knotting of the DNA molecule and by virtue of their nuclear localization sequences (NLS) target the nucleoprotein complex to the nucleus ***(4)***. Eventually the T-strand is converted into a dsDNA molecule, which integrates into one of the chromosomes of the recipient cell as T-DNA. The high-transformation efficiency of this vector system can largely be explained by the specific properties of the virulence proteins, which are translocated into the recipient cells together

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with the T-strand. Yeasts *(5–7)* and fungi *(8,9)* have been added to the list of species that can efficiently be transformed by *Agrobacterium*.

The recipient cell largely determines the fate of the transforming DNA. In plants and most fungi the T-DNA integrates at random positions in the chromosome by nonhomologous recombination. However, in the yeast *S. cerevisiae* the T-DNA integrates preferably by homologous recombination *(5,10–12)*. Transformation of *S. cerevisiae* occurs at a frequency of up to 10^{-4} when integration by homologous recombination is possible or maintenance by extrachromosomal replication through the 2μ plasmid replicon *(5)* or an ARS (autonomous replication sequence) *(7)*. T-DNA vectors lacking homology with the yeast genome give 50- to 100-fold less transformants *(6)* and in that case the integration is mediated by the nonhomologous end-joining machinery of yeast *(10)*. Besides the yeast *Saccharomyces cerevisiae* transformation of the yeast *Kluyveromyces lactis (13)* and numerous fungi *(8,9)* (*see* **Chapter 36**) has been achieved using similar protocols. These protocols involve cocultivation of *Agrobacterium* and recipient cells on a filter placed on a solid medium containing inducing agents for the *Agrobacterium* virulence genes.

In the natural situation the DNA segment which is transferred to plant cells by *Agrobacterium*, the T-region, is located on a 200-kbp plasmid called the Ti plasmid. Virulence genes located elsewhere on the Ti plasmid encode the transport system, a type4 secretion system, by which the T-DNA is translocated into recipient cells *(2)*. The T-region is surrounded by a direct repeat of 24-bp and can be placed on another plasmid *(14)* or on the chromosome *(15)*. Transfer of the T-DNA occurs equally well from such a binary situation with the T-region on another plasmid than the Ti plasmid as when present in cis on the Ti plasmid. In practice wide host range plasmids of incompatibility group P are preferred as T-DNA vectors, which are usually called binary vectors *(14,16)*. As the T-DNA integrates at a fairly random position in the genome, the T-DNA has been used for gene tagging in plants just like transposons. The construction of a small library of T-DNA insertion mutants in *S. cerevisiae* showed that this is feasible also in yeast *(17)*. The T-DNA was found to integrate with higher efficiency by homologous recombination than plasmid DNA in the yeast *Kluyveromyces lactis*, and therefore, the T-DNA may be the preferred vector for gene targeting *(13)*.

2. Materials

2.1. Media (see *Notes 1* and *2*)

1. Luria-Bertani broth (LB): Add 10 g Bacto-tryptone (Difco), 5 g yeast extract (Difco), 8 g NaCl, and 18 g agar to 1 L H_2O (*see* **Note 3**).
2. YPD: Add 10 g yeast extract (Difco), 20 g bacto-peptone (Difco), 10 g glucose, and 18 g agar to 1 L H_2O.

3. YPAD: YPD (1 L) with 100 mg adenine (Sigma).
4. IM (induction medium): Add 0.8 mL K, 20 mL M-N, 5 mL Microspores, 10 mL Fe^{2+} stock, 1 mL Ca^{2+} stock, 2.5 mL NH_4^+ stock, 40 mL MES, pH 5.5, 5.7 mL 87% glycerol (Merck), and 10 mL glucose to H_2O to a final volume of 1 L. For solid IM, add 18 g agar and use 5 mL glucose stock instead of 10 mL (*see* **Note 4**).

2.2. Solutions for Preparation of Media

Prepare the following stock solutions by dissolving in 1 L H_2O.

1. K: Mix 1.25*M* KH_2PO_4 and 1.25*M* K_2HPO_4 to pH 4.8.
2. M-N: 30 g of $MgSO_4 \cdot 7H_2O$ and 15 g of NaCl.
3. Microspores: 100 mg each of Na_2MoO_4, $MnSO_4 \cdot H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, and H_3BO_3.
4. Fe^{2+} stock: 100 mg of $FeSO_4 \cdot 7H_2O$.
5. Ca^{2+} stock: 10 g of $CaCl_2 \cdot 2H_2O$.
6. NH_4^+ stock: 200 g of NH_4NO_3.
7. MES (2-(*N*-morpholino)ethanesulfonic acid): 195.2 g of MES (Sigma), pH 5.5, which results in a 1 *M* stock solution.
8. Glucose: 200 g of D(+)-Glucose.

2.3. Other Solutions

1. Acetosyringone (AS) [3,5-dimethoxy-4-hydroxyacetophenon (Aldrich)]: Make a 1000X stock of 200 m*M* in dimethyl sulfoxide (DMSO).
2. Cefotaxime sodium (Duchefa): Make a 1000X stock of 200 mg/mL in H_2O.
3. Rifampicin (Duchefa): Make a 500X stock of 10 mg/mL in methanol.
4. Kanamycin (Duchefa): Make a 500X stock of 50 mg/mL in H_2O.
5. Geneticin [G418 sulfate (Gibco BRL)]: Make a 1000X stock of 200 mg/mL in H_2O.
6. Physiological salt solution: 0.9 % (w/v) NaCl in H_2O.

2.4. Yeast Recipient Strains

We have performed cocultivation experiments with many yeast strains including derivatives of the *S. cerevisiae* strains RSY12 (*MATa leu2-3, 112 his3-11, 15 ura3Δ::HIS3*), YPH250 (*MATα ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1*) and JKM115 (*MATα Δho Δhml::ADE1 Δhmr::ADE1 ade1 leu2-3, 112 lys5 trp1::hisG ura3-52*) (***18,19***) (*see* **Note 5**).

2.5. *Agrobacterium* Donor Strains

Several *Agrobacterium* strains have been used in cocultivation experiments. In our laboratory we routinely use the *Agrobacterium* helper strains LBA1100 (***20***), LBA1126 (***8***), and LBA1119/EHA105 (***21***). These strains contain a chromosomal rifampicin resistance marker, and in addition the first two strains have a spectinomycin resistance gene on the Ti plasmid (*see* **Note 6**).

2.6. Binary Vectors

Usually the binary vector system *(14)* is used for yeast transformation. In this case the T-DNA is present on a separate plasmid, called the binary vector, in the *Agrobacterium* helper strain. The vectors we have used mostly are derivatives of a vector called pBin19 *(16)*. From this vector we have removed the T-DNA by BglII digestion and reinserted a synthetic piece of DNA with a multiple cloning site that is flanked by border repeats (pSDM14). Introduction of the KanMX marker *(22)* into the HpaI site of pSDM14 led to the binary vector pSDM8000 *(10)*. This vector lacks homology with the *S. cerevisiae* genome.

The same KanMX fragment surrounded by segments from the *S. cerevisiae* genome (from the PDA1 locus) was integrated into the XhoI and KpnI sites of the pSDM14 vector to create pSDM8001 *(11)*. Both pSDM8000 and pSDM8001 have a vector backbone which provides *Agrobacterium* with kanamycin resistance. The KanMX cassette allows selection of transgenic *S. cerevisiae* cells that are resistant to geneticin (G418).

2.7. Supplies for Analysis of Yeast Transformants

1. Sterile toothpicks.
2. Glycerol solution: 50% (v/v) glycerol in H_2O. Sterilize in the autoclave.
3. Polymerase chain reaction (PCR) tubes.
4. A polymerase for PCR, the corresponding 10X PCR reaction buffer and Mg^{2+}-salt solution. These materials can be purchased from many suppliers.
5. Primers for the PCR reaction. The primers are dissolved in H_2O to a final concentration of 100 μ*M* yielding a 100X concentrated solution.
6. Deoxynucleotide triphosphate (dNTP) solution. We use a 100X-concentrated mix of dATP, dCTP, dGTP, and dTTP. The concentration of each dNTP in the 100X-concentrated solution is 25 m*M*. These dNTPs can be obtained from many suppliers.
7. A PCR apparatus and equipment for agarose gelelectrophoresis.

3. Method

3.1. Preparation of Fresh Yeast and Agrobacterium Cultures

Grow *S. cerevisiae* strains on an YPD plate (or an YPAD plate in case of an adenine auxotroph) at 30°C and *A. tumefaciens* strains on an LB plate containing the appropriate antibiotics at 28°C (*see* **Note 7**). For yeast overnight growth will do, but for *Agrobacterium* this requires 3 d. Inoculate the *S. cerevisiae* strains into 10 mL YPD and the *A. tumefaciens* strains into 5 mL LB containing the appropriate antibiotics, and incubate overnight at 30°C and 28°C, respectively, on a shaker.

3.2. Preparation of A. tumefaciens Before Transformation

1. Pellet cells from 2 mL *A. tumefaciens* overnight culture by centrifugation for 3 min at 15,000*g*. Remove the supernatant and resuspend the cells in 1 mL of freshly prepared IM to wash.

2. Pellet cells again by centrifugation, remove supernatant and resuspend the cells in 200 µL IM.
3. Transfer cells to 5 mL IM containing 0.2 m*M* AS (to an OD_{600} of 0.25) and incubate for 5 to 6 h at 28°C on a shaker.

3.3. Preparation of *S. cerevisiae* Before Transformation

1. Add 5 mL *S. cerevisiae* overnight culture to 50 mL YPD prewarmed to 30°C and incubate for 5 to 6 h at 30°C on a shaker.
2. Harvest the cells in a sterile 50-mL disposable centrifuge tube by centrifugation for 5 min at 3000*g*.
3. Remove supernatant and resuspend the cells in 25 mL IM to wash.
4. Pellet cells again by centrifugation. Pour off the supernatant and resuspend the cells in 0.5 mL IM. The OD_{600} of the suspension is about 4.

3.4. Preparation of IM and Filters Before Transformation

1. Prepare IM plates containing 0.2 m*M* AS and amino acids and/or nucleotides as required to complement the auxotrophic requirements of the yeast strains used.
2. Cut round cellulose nitrate filters (Sartorius) into four equal pieces and place three or four sterilized pieces of a filter on the medium. Alternatively, cut rectangular pieces from sheets (Schleicher & Schuell) (*see* **Note 8**).

3.5. Cocultivation

1. Transfer 60 µL of *A. tumefaciens* culture to a sterile 1.5-mL Eppendorf tube. Add 60 µL of the slurry of *S. cerevisiae* cells and mix to form the cocultivation mix (*see* **Note 9**).
2. Carefully spot 100 µL of cocultivation mix onto a cellulose nitrate filter placed on the IM plate containing 0.2 m*M* AS and incubate for 3 to 9 d at 22°C (*see* **Notes 10** and **11**).

3.6. Recovery of Transformants

1. Transfer the filter with the cocultivation mix after 3 to 9 d cocultivation to a 2-mL Eppendorf tube containing 2 mL sterile physiological salt solution and vortex vigorously to resuspend the cells.
2. Use 10 µL of the suspension and dilute 10^5- to 10^6-fold in physiological salt solution.
3. Plate 50 to 100 µL of these dilutions onto YPD plates containing 200 mg/L cefotaxime in order to determine the output number of yeast cells.
4. Plate 50 to 100 µL of these dilutions onto LB plates containing 20 mg/L rifampicin and 100 mg/L kanamycin in order to determine the output number of *A. tumefaciens* cells.
5. Plate out 200 µL of the cocultivation mix/YPD plate containing 200 mg/L cefotaxime and 200 mg/L G418, when a T-DNA is transformed with the KanMX marker that integrates into the genome by nonhomologous recombination. However, plate 50 to 100 µL of a 50-time dilution of the cocultivation mix on two of these selection plates when a T-DNA is transformed with the same marker that

Table 1
Frequencies of T-DNA Integration After Co-cultivating *Agrobacterium tumefaciens* and *Saccharomyces cerevisiae*

Transformation	Co-cultivation period (d)	G418 -resistant colonies Number	Frequency
LBA1119 (pSDM8000) × YPH250	3	10	3×10^{-8}
LBA1119 (pSDM8000) × YPH250	9	58	2×10^{-7}
LBA1119 (pSDM8000) × JKM115	9	834	1×10^{-5}
LBA1119 (pSDM8001) × YPH250	3	720	2×10^{-6}
LBA1119 (pSDM8001) × YPH250	9	10,233	3×10^{-5}
LBA1119 (pSDM8001) × JKM115	9	9100	6×10^{-5}

integrates by homologous recombination. Use appropriate selection plates consisting of a minimal yeast medium if the T-DNA contains an auxotrophic marker such as *URA3* or *LEU2* instead of the KanMX marker. The NatMX marker has also been used successfully.

6. Incubate the plates at 30°C for 3 to 5 d until colonies appear.
7. Determine the number of transformants and output cells. Divide the number of transformants by the number of output cells to determine the frequency of T-DNA integration.
8. Table 1 shows the results of transformation experiments with the *S. cerevisiae* strains YPH250 and JKM115, and the *A. tumefaciens* strains LBA1119 (pSDM8000), and LBA1119 (pSDM8001), respectively.

3.7. Molecular Analysis of Transformants

3.7.1. Colony Purification of Yeast Transformants

1. Yeast transformants are colony purified by streaking them on a selective plate followed by incubation for approx 2 d to obtain loose colonies. Usually a single purification step is sufficient.
2. Two colonies originating from a single primary transformant are selected. These colonies are picked and grown overnight in liquid selective medium.
3. Aliquots of the culture are frozen at –80°C after the addition of sterile glycerol until a final concentration of 15 to 25 %.

3.7.2. Molecular Analysis of the T-DNA Integration

1. A correct integration of the T-DNA is checked by PCR and/or Southern blot analysis. For the initial analysis PCR is the preferred method (*see* **Note 12**).
2. Using a sterile toothpick take a small amount of cells, approx 2 µL, and streak the cells to the wall of a PCR tube (*see* **Note 13**).
3. Add the components for a regular PCR reaction; we usually use a final volume of 50 µL. For a 50 µL volume add (in this sequence): H_2O, 5 µL of 10X concentrated

PCR buffer, 0.5 μL dNTP mix, 0.5 μL of each of the two primers, $MgCl_2$ or $MgSO_4$ (final concentration as recommended by the supplier of the polymerase), and finally the polymerase (2.5 units). Vortex well and keep the tubes in ice or start the PCR reaction immediately.

4. Place the tubes in the PCR apparatus (hot start) and start the program. Depending on the annealing temperature of the primers and the expected length of the PCR fragment, adjust the PCR program. For a typical PCR reaction we use the following program: Step 1, 3 min at 94°C; step 2, 1 min at 58°C; step 3, 2 min at 74°C; step 4, 1 min at 94°C; repeat 30 times steps 2 to 4; step 5, 1 min at 58°C; step 6, 10 min at 74°C; step 7, refrigerate.
5. Analyse an aliquot of the PCR reaction (approx 10 μL) by agarose gelelectrophoresis and ethidium bromide staining.

4. Notes

1. Media and solutions are sterilized in the autoclave (20 min., 120°C) or by filter sterilization (e.g., antibiotics, glucose, MES).
2. We use Micro agar (Duchefa) or Select agar (Gibco BRL) for solidifying the medium.
3. The H_2O we use is de-ionized and further purified on a MilliQ column.
4. The amount of glucose is lower in the solid cocultivation IM medium than the standard IM, because too much sugar leads to overgrowth of the yeast and lower T-DNA transfer.
5. Several different haploid and diploid yeast strains (including YPH250, S288C, W303, and BY4743) have been used for transformation by *Agrobacterium*. We have not come across strains that could not be transformed. Mutants with purine auxotrophy (ADE1-8) seem more efficiently transformed than the corresponding wild types (*23*). To see this effect the purine must be omitted from the cocultivation plates. For plants it has been reported that the addition of purine synthesis inhibitors (such as azaserine, acivicine) during cocultivation leads to enhanced transformation (*23*). We have tested this for yeast, but have not seen a significant effect.
6. The *Agrobacterium* virulence system can also be used for the delivery of specific proteins into yeast (*24*). This has been used to deliver the Cre recombinase to mediate site specific recombination reactions in the genome of yeast (*3,24*).
7. *Agrobacterium* strains with binary vectors are best grown on a medium that is selective for maintenance of the binary vector as such binary vectors usually are not completely stable.
8. 96-well microtiter plates can be used for large scale transformation on rectangular cellulose nitrate filters of the same size as the microtiter plate. This becomes useful when analyzing the complete collection of yeast (deletion) mutants.
9. Transformation can also be accomplished by replica plating yeast colonies directly to a cocultivation plate with *Agrobacterium* (*23*).
10. The minimum cocultivation time of 2 d gives only few transformants. The number of transformants, however, increases steadily during the 9 d of cocultivation.
11. The cocultivation temperature needs to be controlled. We obtain the best results with incubations at about 22°C.

12. For analysis of transformants obtained by homologous recombination one PCR primer is designed for annealing to yeast DNA sequences just upstream of the expected integration site and one primer is designed for annealing to the T-DNA insert. The annealing sites for these primers are preferentially chosen to yield PCR products of less than 1-kb. In a similar way the other site of the integration has to be checked. We design primers with an annealing temperature of at least 60°C. The correct integration can also be checked by Southern blot analysis. For this technique chromosomal DNA is digested preferentially with restriction enzymes cutting just upstream and downstream of the integration sites. Both a DNA probe consisting of sequences flanking the expected integration site and sequences of the T-DNA insert are used. To determine the chromosomal location of the T-DNA integrated by nonhomologous integration more laborious methods have to be used. For this purpose we successfully used vectorette PCR and TAIL-PCR (*10*).
13. Although PCR analysis on cells often is very successful, sometimes PCR analysis fails because of interference by cellular components. Alternatively, isolated chromosomal DNA can be used as template.

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Agrobacterium Protocols

Second Edition, Volume 2

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Rapid changes and significant progress have been made in the use of *Agrobacterium* to genetically transform plants for both basic research purposes and agricultural development. In Agrobacterium *Protocols, Second Edition, Volumes 1 and 2,* a team of leading experts and veteran researchers describe in detail their best techniques for delivering DNA to plant cells and permanently altering their genomes. Volume 1 details the most updated techniques available for twenty-six plant species drawn from cereal crops, industrial plants, legume plants, and vegetable plants, and presents various methods for introducing DNA into three major model plant species, *Arabidopsis thaliana, Medicago truncatula,* and *Nicotiana.* The authors also outline the basic methods in *Agrobacterium* manipulation and strategies for vector construction, major components of plant transformation that are often neglected by many plant biologists. Volume 2 contains another thirty-three proven techniques for root plants, turf grasses, woody species, tropic plants, nuts and fruits, ornamental plants, and medicinal plants. Additional chapters provide methods for introducing DNA into non-plant species, such as bacteria, fungi, algae, and mammalian cells. The protocols follow the successful *Methods in Molecular Biology™* series format, each offering step-by-step laboratory instructions, an introduction outlining the principles behind the technique, lists of the necessary equipment and reagents, and tips on troubleshooting and avoiding known pitfalls.

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