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Mammalian Cells

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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 × 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.

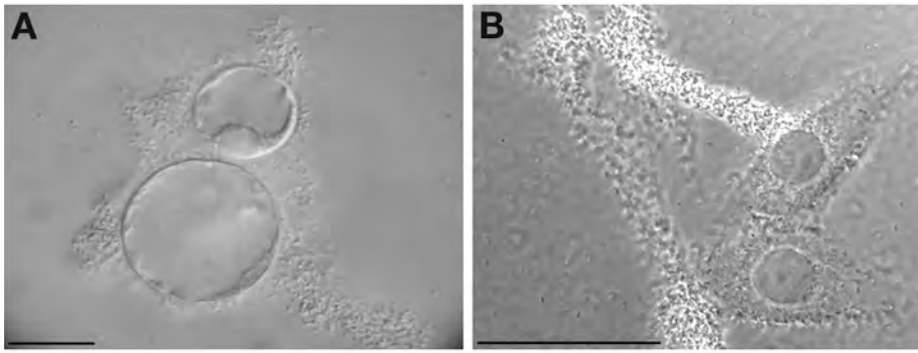

Fig. 1. *Agrobacterium* cells are capable of attaching to cells of evolutionary-distant host species. Similar attachment patterns, with characteristic bacterial aggregates at and around the host cells, were observed when *Agrobacterium* was co-incubated with petunia protoplasts **(A)** and HeLa cells **(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

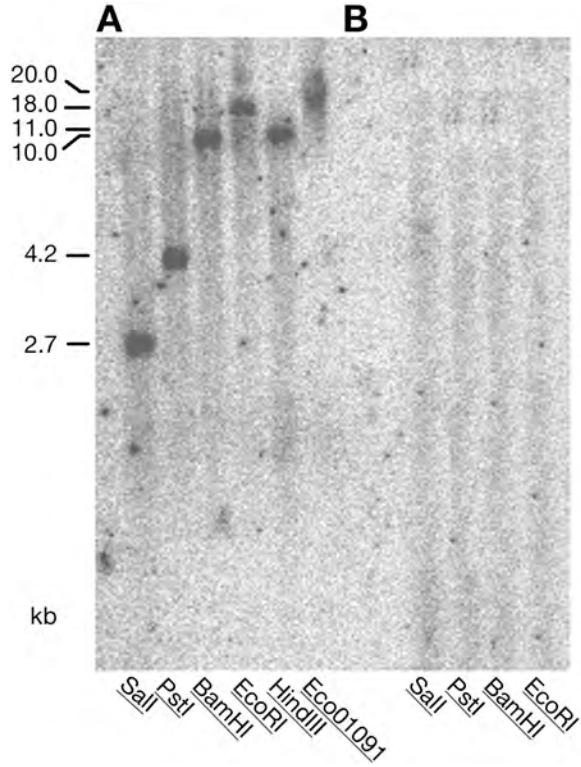

Fig. 2. Southern blot analysis of a transgenic HeLa cell line. Genomic DNA was extracted from cell cultures derived from a cell line stably-transformed with the pNeo plasmid, and purified DNA was digested with the indicated restriction endonucleases. *Pst*I, *Bam*HI, *Eco*RI, and *Hind*III cut once inside 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.

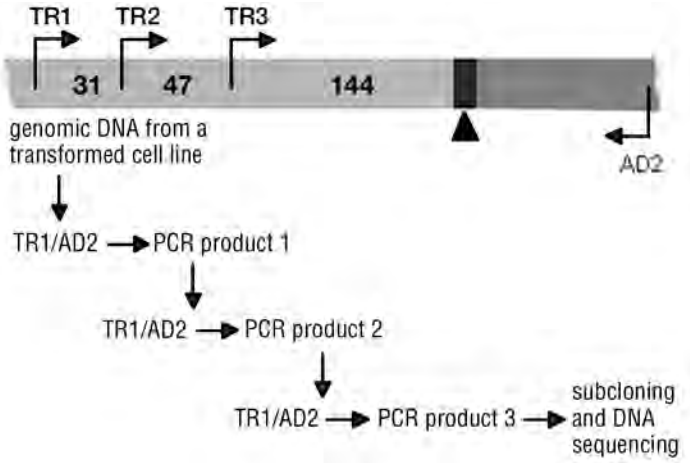

Fig. 3. A basic strategy for TAIL-PCR-based amplification of T-DNA-host DNA integration junctions. Three nested forward primers (TR1, TR2, and TR3), designed to reside within 200- to 300-bp from the T-DNA right border, are used in three consecutive PCR reactions with a degenerate reverse primer (AD2) 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-

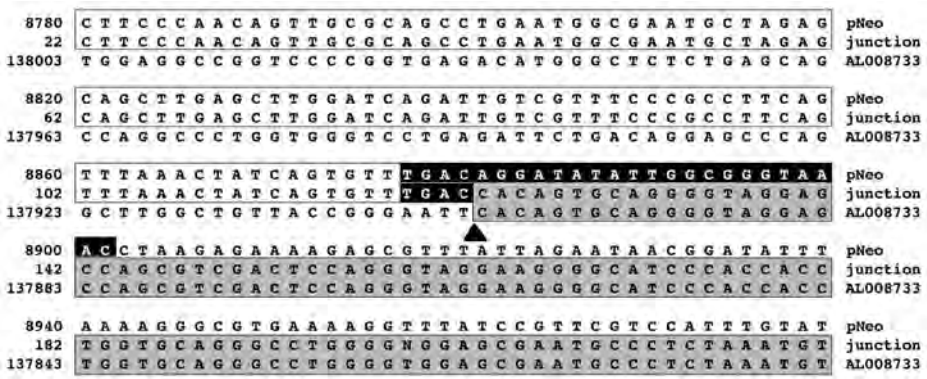

Fig. 4. Nucleotide sequence alignment of the right T-DNA border region of pNeo, the isolated integration junction from an *Agrobacterium*-transformed HeLa cell DNA, and the human genomic DNA Accession No. AL008733. All sequences are shown in the 5′ to 3′ direction. The pNeo sequence is based on the right 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.

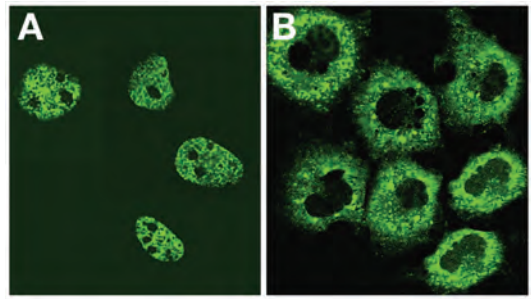

Fig. 5. Expression of GFP-tagged *Agrobacterium* proteins in mammalian cells. The T-strand associated bacterial proteins, known to interact with different host plant factors (***28,42***), differ in their intracellular localization in mammalian cells. While VirD2 efficiently localizes within the mammalian 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-

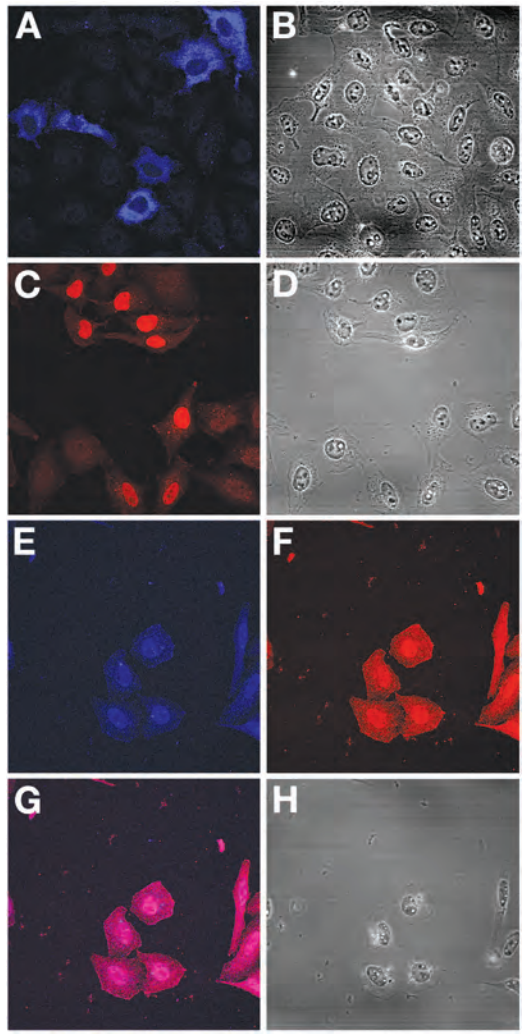

Fig. 6. Indirect immunofluorescence detection of *Agrobacterium* and plant proteins expressed in mammalian cells. Myc-tagged VirE2, similarly to GFP-tagged VirE2 (*see* **Fig. 5**), is not recognized by the nuclear import apparatus of mammalian cells and remains cytoplasmic as detected using primary 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.

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To Jeff Schell (1935–2003), one of my Ph.D. mentors, for his 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 tranformation 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 co-cultivation 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 Volume II, there are a total of eight parts. Parts III through XIII collect 61 chapters covering protocols for 59 plant species. 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. Volume II contains seven of the eleven parts (Part VII to XIII) of plant protocols. Part XIV (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.

This book 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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