

Plant Transformation

Agrobacterium-Mediated Gene Transfer

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Summary

Plant transformation is the process by which DNA is introduced into plant cells or tissues. The DNA can come from virtually any source. Gene transfer methodology has become part of an essential technology to manipulate plants for both scientific and commercial purposes. Transgenic plants, the products of this methodology, are useful for dissecting the mechanism(s) of plant gene regulation. This technology is also useful for identifying and evaluating agriculturally useful traits (genes) as well as for their introduction into commercially valuable crops. One of the most efficient methods for gene transfer employs *Agrobacterium tumefaciens* and takes advantage of the naturally evolved crown gall-inducing mechanisms of DNA transfer present in this common soil pathogen. Much has been learned about the mechanisms of this form of DNA movement and subsequent crown gall induction. This information has been applied to develop methods that result in the formation of gall-free, genetically transformed plants. This chapter describes a detailed protocol for *Agrobacterium*-mediated transformation of tobacco cells and their subsequent selection and regeneration into transgenic plants.

Key Words: *Agrobacterium tumefaciens*; genetic selection; plant transformation; regeneration; transgenes, transgenic plants.

1. Introduction

The production of transgenic plants involves the marriage of two critical yet distinct basic technologies. The first directs the introduction of new genetic material into plant cells (transformation); whereas the second uses methods based in tissue culture to regenerate the resulting transformed cells into transgenic plants. Of the various methods developed to introduce DNA into plant cells, most include a transformation step that is mediated by *Agro-*

bacterium tumefaciens (1–4). In nature, *Agrobacterium tumefaciens* is the causative agent of crown gall disease and was discovered at the turn of the last century. However, approx 75 yr passed before it was determined that this ubiquitous soil microorganism is capable of interkingdom DNA transfer (5). The crown gall (tumor) represents a manifestation of the transfer and expression of bacterial DNA in plant cells. This highly evolved and elegant mechanism of transforming plant cells has been harnessed by plant biotechnologists for both knowledge and profit. For the purposes of this chapter, we highlight some of the salient features of *Agrobacterium*-mediated transformation of plant cells and their regeneration into transgenic plants as these features need to be understood from a basic perspective to carry out effectively the procedures presented. However, this is not an extensive review of the subject and the reader is urged to read recently published reviews on the use of *Agrobacterium* as a vector for gene transfer (1–5) and on *Agrobacterium* as an agent of disease (6).

Agrobacterium is attracted to the amino acids, sugars and organic acids that are released from wounded plant tissues. It responds to these chemoattractants by seeking out the wounded cells that produced them and then by binding to them by a polar attachment mechanism (4,7). During attachment, coordinated expression from a suite of genetic operons critical to the gene transfer process also begins (8). These operons—*virB*, *virC*, *virD*, *virE*, and *virG*—are collectively termed the “*vir* regulon,” and are coordinately regulated by a *virA/virG* two-component system. The wound phenolics and monosaccharides directly or indirectly cause the autophosphorylation of the *virA* transmembrane receptor kinase, which in turn activates the soluble cytoplasmic transcriptional factor *virG* through another phosphorylation event. Activated *virG* subsequently stimulates the transcription of the individual *vir* operons by binding to the upstream “*vir* box” *cis*/enhancer elements (2,7).

Gene products that are generated from transcription of the *vir* operons perform functions that are critical to the transfer of a DNA fragment called T-DNA from the tumor-inducing (Ti) plasmid localized in the bacteria into plant cells. The gene products *virD1* and *virD2* are cooperatively responsible for cleavage of the T-strand delimited by the presence of border sequences (right and left border) (8). The *virD2* protein binds covalently to the 5'-end of the T-strand which is then coated to form a T-complex with the single strand binding protein *virE2* either in the bacteria or *in planta* (9). This T-complex is exported via a type 4 bacterial secretion system encoded by the *virB* operon and *virD4* (9). Both *virD2* and *virE2* contain nuclear localization sequences that interact with the plant components that include an importin- α , a type 2C protein phosphatase and three cyclophilins (*virD2*-interacting factors), and *vip1* and *vip2* (*virE2*-interacting factors) which together help target the T-complex into the plant nucleus (4,5). Once inside the nucleus the T-strand is integrated into the plant

genome via nonhomologous recombination mediated by plant encoded proteins that are likely part of recombination and/or repair process in plants (10,11).

Instead of the naturally occurring single Ti plasmid, most laboratory strains of *Agrobacterium* used for transformation employ a binary system consisting of two plasmids (12). One plasmid contains the *vir* regulon sequences, the gene products of which work in *trans* to transfer the T-DNA from a separate plasmid. The oncogenes (gall-forming sequences) have been removed from the T-DNA and in their place engineered expression cassettes with genes from virtually any source may be substituted, usually by convenient insertion into multiple cloning sequences that have been incorporated into these plasmids. Different strains of *A. tumefaciens* display different levels of virulence (transformability), much of which stems from differences in the *vir* sequences (13).

Once a plant cell has incorporated the introduced DNA in a stable manner (i.e., covalently integrated within the host plant's genome), the next step is to regenerate a plant from the transformed cells. Position, frequency, and scope of regeneration events are critical to the isolation of transgenic plants (14). Most often, the major limiting step in the isolation of transgenic plants is a lack of regeneration occurring from within the transformed cell populations. There is a large amount of variability in the frequency and scope of regeneration among different angiosperm species as well as among different cultivars of any one species (15). The two pathways of regeneration that have been observed in most angiosperms are organogenesis and somatic embryogenesis (for review, see ref. 16). Organogenesis involves the regeneration of adventitious shoots or roots through the formation of organized, meristematic tissues. The second pathway involves the formation of embryos or embryo-like structures from somatic tissues. It has been suggested that somatic embryogenesis and organogenesis reflect different developmental events that are most likely mutually exclusive (16). This chapter presents a method for the organogenic regeneration of tobacco plants from leaf discs following *Agrobacterium*-mediated transformation that is loosely based on a landmark paper published nearly 20 yr ago (17).

2. Materials

Unless stated otherwise, all reagents and chemicals used in this protocol were of high purity and were analytical grade and/or tested for molecular biology or plant cell tissue culture applications. The water used was deionized and filtered through a Nanopure (Barnstead, Dubuque, IA) water purification system.

2.1. Supplies and Equipment

1. GA7 tissue culture boxes with lids (Magenta or equivalent).
2. Laminar flow hood.

3. Forceps.
4. Scalpels.
5. Sterile, disposable Petri dishes.
6. Sterile filter paper.
7. Cork borers (0.7 mm).
8. Cork borer sharpener.
9. Bunsen burners.
10. Inoculation loops.
11. Environmental shaker incubators (25°C).
12. Environmental growth chambers.
13. P20, P200, P1000, and P5000, Pipetman (or equivalent) micropipettors and appropriate tips.
14. 1.5-mL Microfuge tubes.
15. 15- and 50-mL capped centrifuge tubes (Falcon or equivalent).
16. 15% (v/v) Household bleach.
17. 70% Ethanol.
18. Laboratory sealing film (Parafilm or equivalent).
19. Heated water bath at 55°C.
20. Disposable 10-mL sterile syringes.
21. Acrodisc 0.2- μ m syringe filter sterilization units (or equivalent).

2.2. Reagents, Solutions, and Media

1. 1/2X MSO, pH 5.8: half-strength Murashige and Skoog (MS) medium (**18**) solidified with 0.8% Phytagar (Invitrogen, Carlsbad, CA) (*see Note 1*).
2. *Agrobacterium* strains: any one of several common disarmed (non-gall-forming) laboratory strains (e.g., EHA 101, 105, C58, and LBA4404) containing an engineered binary transformation vector (*see Note 2*).
3. YEP medium, pH 7.2: 5.0 g/L of Bacto-yeast extract, 10.0 g/L of Bacto-peptone, 10 g/L of NaCl, 15 g/L of Bacto-agar.
4. Filter-sterilized MS20IM *Agrobacterium* induction medium, pH 5.25: MS salts and vitamins supplemented with 2 % (w/v) sucrose, 100 μ M acetosyringone, 1 mM betaine phosphate or proline, and 2.5 mM 2-(4-morpholino)ethanesulfonic acid (MES) (*see Note 3*).
5. Cocultivation medium, pH 5.8: MS medium supplemented with 4.5 μ M benzylaminopurine (BA), 0.5 μ M naphthalene acetic acid (NAA) and solidified with 0.8% (w/v) Phytagar (Gibco).
6. MSBN1.1 shoot regeneration medium, pH 5.8: identical to the cocultivation medium shown above with the exception that selective agents are used as appropriate (*see Note 4*).
7. MSHF rooting medium, pH 5.8: MS medium solidified with 0.8% Phytagar and supplemented with selective agents when appropriate.

3. Methods

3.1. Growth and Propagation of Tobacco (*Nicotiana tabacum*)

Most plants offer a number of tissues that will regenerate under the proper conditions. However, efficiencies may vary greatly. Plants or regenerable plant tissues grown under axenic conditions in culture offer the most consistent results with respect to regeneration, as some of the environmental conditioning that varies with season in plants grown outside of the laboratory has been eliminated. Material from cultures also leads to fewer downstream contamination problems. Shown below is a procedure for growing tobacco plants under axenic conditions. It should be noted that the methods have been optimized for the cultivar “Xanthi”; however, others, such as “SR1” also have been successfully transformed using this procedure.

1. Surface sterilize tobacco seeds by placing them in 15-mL conical centrifuge tubes and filling them with 10 mL of a 15% bleach solution plus one drop of Tween-20.
2. Shake the tubes continuously for 15 min on a gyratory shaker at 110 rpm.
3. Allow the seeds to settle, pipet off the Clorox solution, and rinse three times with sterile distilled water. Rinsing is accomplished by filling the centrifuge tube with 10 mL of sterile distilled water, then allowing the seeds to settle and pipetting off the rinse water. Remove all but 1 mL of water during the final rinse.
4. Dispense the last milliliter of water with seeds using a pipet onto 100 × 20 mm Petri dishes containing 25 mL of agar solidified 1/2X MSO.
5. Incubate plates at 26°C under soft fluorescent lights with a 16-h photoperiod.
6. After 10–14 d, transfer germinating green seedlings to Magenta boxes containing 50 mL of autoclaved MSHF (see Note 5).
7. Plants may be multiplied by removing expanded leaves from rooted plants, cutting the remaining stem between nodes, and inserting the resulting stem pieces into Magenta boxes containing MSHF. Individual plants may be maintained indefinitely without multiplication by simply propagating the shoot tip in a similar manner. Repeat subcultures to fresh medium once every 4 wk.

3.2. Growth of *Agrobacterium* and Preparation of Inoculum

Compared to other laboratory strains of bacteria such as *Escherichia coli*, *Agrobacterium* grows relatively slowly. To grow overnight cultures of sufficient densities consistently and conveniently, it is important to inoculate them with cells actively growing on solid medium.

1. Prepare a 50-mL culture tube containing 10 mL of YEP media containing the appropriate selective antibiotics.
2. Inoculate the tube with one loopful of active bacteria (*A. tumefaciens* containing a binary vector with the gene[s] of interest) taken from a selection plate kept at 4°C (see Note 6).

3. Grow 20–24 h at 25°C with agitation of 100–150 rpm. If an environmental shaker is unavailable, room temperature should be sufficient.
4. Determine the optical density of the cultures spectrophotometrically at 420 nm. Calculate the amount of culture needed to provide an optical density of 0.5 when diluted to 20 mL.
5. Centrifuge the appropriate amount of culture in a 50-mL Falcon tube for 15 min at 2500g.
6. Pour off the supernatant
7. Resuspend the pellet in 20 mL of MS20IM medium
8. Induce the *Agrobacterium* for transformation by shaking on a rotary shaker (100–150 rpm) for 5 h at 20–25°C (room temperature).

3.3. Preparation and Infection of Leaf Disks

The overall objective in preparing plant material is to maximize the number of wounded, cut surfaces for *Agrobacterium* attachment while maintaining enough healthy tissue that will later support efficient regeneration.

1. Remove expanded leaves from rooted plants growing axenically in culture and float them in 100-mm Petri dishes containing sterile MS20IM.
2. Cut disks from the leaves in dishes under MS20IM using a flame-sterilized 0.7-cm cork borer. Prepare leaf disks in batches of approx 50/plate (see **Note 7**).
3. Set aside approx 16 leaf disks to serve as controls for the transformation/regeneration procedure by transferring them directly to 100 × 15 mm Petri dishes containing cocultivation medium overlaid with sterile filter paper (8 disks/plate) after gently blotting away excess MS20IM using sterile filter paper.
4. Decant the MS20IM from the plates containing the remaining leaf disks using a sterile pipet and replace it with induced *A. tumefaciens* suspension. Incubate at room temperature (approx 25°C) for 10–20 min with occasional swirling.

3.4. Cocultivation

Agrobacterium attachment to plant tissue is completed during the earlier stages of cocultivation. The physical transfer of genetic material occurs later.

1. Remove each disk individually, gently blot off excess culture using sterile filter paper, and transfer to 100 × 15 mm Petri dishes containing cocultivation media overlaid with sterile filter paper. Place about 16 disks/plate.
2. For large scale experiments we routinely cut about 800 disks and inoculate Petri dishes with approx 24 disks/plate.
3. Seal all Petri dishes with laboratory sealing film (Parafilm or equivalent)
4. Incubate cultures at 20°C in the dark for 3 d (see **Note 8**).

3.5. Selection and Regeneration of Transgenic Tobacco Shoots

Several important events occur during selection and regeneration. Antibiotic(s) that do not affect plant cells are used to eliminate or arrest the growth of *A. tumefaciens*. Conditions are also optimized for the adventitious,

organogenic regeneration of new plant tissues. To enrich the population of new growth with transgenic tissues, additional selective agents are incorporated into the regeneration medium for the purposes of genetic selection (*see Note 4*). Genetic selection is the process of selecting preferentially for those cells that have been transformed by the incoming transgenes. A selective advantage can be conferred on the transformed cells through the introduction of genes encoding antibiotic resistance or resistance to some metabolic inhibitor such as a herbicide. In the presence of the antibiotic or herbicide, the untransformed cells die whereas the transformed cells grow and multiply. If no form of genetic selection were used, then one would be faced with the option of screening every shoot that regenerated in a transformation experiment. In cases where the transformation frequency is high (i.e., the number of transformed cells or shoots arising from an explant), this would be feasible. However, for other species with lower transformation frequencies, this would become a laborious if not impossible task. Therefore, genetic selection is an essential component of any plant transformation protocol and has been accomplished by using various marker genes (*14,16*).

1. Subculture the disks to selective medium. All those infected with *A. tumefaciens* and half of the control disks (no infection with *A. tumefaciens*) should be transferred to MSBN1.1 regeneration medium containing the appropriate selective agents in 100 × 15 mm Petri dishes. The control disks under these conditions will provide an indication of nontransgenic regeneration (“escapes”) under selection. Transfer the remaining control disks to regeneration medium (MSBN1.1) containing only the selective agent used to eliminate *Agrobacterium* (this is a control to evaluate overall regeneration frequency). In all cases, plate at a density of approx 8 disks/plate.
2. Maintain cultures at 20°C in low light (approx 45 μE/m²s). Check regularly for contamination. If contamination is discovered, unaffected disks within the plate may be subcultured to fresh MSBN1.1.
3. All disks should be subcultured to fresh selection plates every 2–3 wk. The disks will expand and develop callus over time. Try to ensure that the expanded disks establish good contact with the media. Shoots will appear in 3–4 wk.

3.6. Rooting of Transgenic Shoots to Recover Complete Plantlets

The next step is to recover complete plants from any regenerated shoots through root organogenesis. In addition, the first meaningful screen to test for transformation is often the rooting procedure, as root organogenesis is usually more sensitive to the incorporated selective agents than shoot regeneration. Shoots recovered from selective regeneration procedures that do not root under selection are rarely transgenic and should be discarded.

1. Carefully remove regenerated shoots by cutting them at their base using a sterile scalpel and forceps and place them in GA7 boxes (about four shoots per vessel)

containing 50 mL of MSHF supplemented with selective agents. Roots should become visible within approx 10 d (*see Note 9*).

2. Subculture only the shoots that have rooted by cutting off the shoot with the top four internodes and introducing these individually into a GA7 box containing 50 mL of MSHF supplemented with the appropriate selective agents. These individual shoots may be considered as putative transformants.
3. Rooted shoots can be maintained and/or propagated to establish individual lines at monthly intervals as described in **Subheading 3.1**. Alternatively, the plants may be acclimatized and transferred to the greenhouse to produce seeds. It takes about 3 mo to set seed, depending on conditions.

3.7. Analysis of Transgenic Plants

Recovered plants are typically analyzed on a number of different levels to determine that they are in fact transgenic. Once plants grow large enough to provide enough tissue for analyses without compromising health, they may be assayed for transgene expression and molecularly for the presence of the appropriate sequences. The assay for gene expression is conducted using methods consistent with the transgene coding sequence and desired results. If such a procedure is impossible or inconvenient, polymerase chain reactions (PCRs) may also be performed. Plants that give a positive result must then be analyzed using a DNA blotting procedure (Southern) to confirm the presence of transgenes and their abundance (*see Note 10*).

4. Notes

1. Premixed tissue culture reagents are available commercially from a number of different sources. We routinely purchase MS salts and vitamins as a powder or concentrated stock solution from either Gibco or Sigma. Reagents from both sources provide consistent results.
2. When selecting a strain of *Agrobacterium* for the purpose of transformation, the genetic background is a factor that should be considered. Although it is well known that most dicot plants are susceptible to *A. tumefaciens* (**13**), resistance of the target plant tissues to this pathogen could be an important factor influencing its virulence and, ultimately, affect the efficiency of plant transformation. A growing body of evidence indicates, for most of the widely used strains of *A. tumefaciens*, wide variations in virulence that depends on the target plant tissue used (**13**). Many of these differences may stem from differences in interactions between the host plant and bacterial *vir* gene products.
3. This medium has been developed to provide *A. tumefaciens* optimal conditions for virulence induction. Environmental factors such as pH, temperature, and osmotic conditions strongly influence the expression and induction of virulence genes (**14**). The most direct effects on virulence induction are mediated by the presence of phenolic compounds such as acetosyringone (3',5'-dimethoxy-4'-

hydroxyacetophenone), sinapinic acid, coniferyl alcohol, caffeic acid, ethyl ferrulate, and methylsyngic acid, which are known inducers of virulence genes in *Agrobacterium* (reviewed by Kado [19]). The virulence induction is also influenced by the presence of other compounds such as monosaccharides (20) and opines (21). Betaine, proline, and other osmoprotective compounds have been shown to enhance synergistically the effect of phenolic compounds (15,22). Betaine has been shown to increase the expression of several virulence genes in *Agrobacterium* (15). Proline or betaine may help the bacteria to adapt to rapid changes in pH and osmotic pressure caused by the proximity of wounded plant cells, thus increasing the transformation efficiency (22).

4. Selective agents used for this purpose are usually prepared as stock solutions that are typically 500- to 1000-fold more concentrated than their working strength in cultures. They may be stored as filter-sterilized solutions in a freezer (-20°C) for up to 2 mo. Shown are the working concentrations (milligram/liter) of several antibiotics routinely used for selection during plant transformation procedures: kanamycin—100; tetracycline—5; gentamicin—20; cefotaxime—250–500; and carbenicillin—500. Kanamycin is commonly used to select for transgenic plant cells and tissues whereas the others are used to select for engineered strains of *A. tumefaciens* (tetracycline and gentamicin) or eliminate it (cefotaxime and carbenicillin) from cultures.
5. Growth of tobacco can vary widely depending on the cultivar and growth conditions. It may be advisable to use a larger culture container to allow for a reasonable amount of time to pass before it becomes necessary to subculture, or to maximize the leaf material available as source tissue for a transformation procedure. We routinely use glass household canning jars containing 100 mL of medium. The plants perform best if the vessels are capped with a sterile plastic cap. Avoid a glass cap and instead use, for example, the bottom of a disposable Petri dish and seal to the container with Parafilm (or equivalent).
6. To ensure that overnight cultures obtain an adequate cell density, it is important to use active inoculum. We routinely maintain the cultures as streaked bacteria on selective plates containing solidified YEP medium. The plates are incubated for approx 48 h at 28°C and then kept in a refrigerator (4°C). The bacteria should be subcultured to fresh plates every 4 wk.

As an alternative to using a sterile loop to streak plates and inoculate liquid cultures, we routinely use sterile pipet tips. The barrel and ejector of the pipettor are sprayed with 70% ethanol and allowed to dry in a laminar flow hood. The pipettor is then used to place bacteria on a pipet tip that can then be used to streak a plate or ejected into a culture tube containing growth medium.

7. As an alternative to using a cork borer to prepare discs, the leaf tissue also may be cut into small squares with a scalpel and forceps. In either case, it is important to be as gentle as possible, because unnecessary wounding may lower regeneration frequencies. In addition, excessive drying may also result in adverse effects. Therefore, it is important to work quickly and minimize exposure of the leaf tissue to open air as much as possible.

8. We have observed that transformation frequencies trend upwards with increasing cocultivation time, up to 5 d. However, overgrowth of *A. tumefaciens* and subsequent losses of plant material owing to contamination result in cocultivation times exceeding 3 d. Overgrowth problems are the result of an interaction between inoculum concentration, cocultivation time, and plant species or cultivar. Therefore, concentration and time should be considered variables for optimization when establishing a transformation system.
9. If one of the transgenes contained within the binary vector is a scoreable marker, it may be possible to conduct a convenient preliminary screen for transformation prior to placing the shoots into rooting medium. After excising the regenerated shoots from the original explant, a very small piece of stem tissue may be taken from the basal region before it is placed in rooting medium. The cutaway stem tissue may then be used to assay for the expression of the scoreable marker. Decisions about moving forward with the corresponding shoots may then be conducted in a more informed manner.
10. It is important to confirm stable incorporation of the introduced gene(s) and its expression in the putatively transformed plants and their siblings. This is possible only if the incorporated DNA has been integrated into the genome of the transformed plant. In annual plants such as tobacco described here, this can be determined easily by backcrossing or selfing the plant to determine if the introduced gene is heritable. In the case of perennial species, often the long generation time makes this type of analysis impractical. Alternatively, transformation can be confirmed through a rigorous and comprehensive Southern analysis of the transformed tissue. Typically this analysis should be performed to reveal and identify different segments of the inserted T-DNA, that is, the presence of both internal and border fragments (23).

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