

Agrobacterium Protocols

Second Edition

Volume 1

Edited by

Kan Wang

***Agrobacterium* Protocols**

METHODS IN MOLECULAR BIOLOGY™

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Volume 1

Edited by

Kan Wang

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and Department of Agronomy,
Iowa State University, Ames, Iowa*

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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 a 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 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. Part I in Volume 1 (*Agrobacterium* Handling) provides six chapters describing basic techniques in *Agrobacterium* manipulation and strategies for vector construction, major components of plant transformation that are often neglected by many plant biologists. Part II in Volume 1 (Model Plants) consists of seven chapters describing various ways to introduce DNA into three major model plant species, *Arabidopsis thaliana*, *Medicago truncatula*, and *Nicotiana*. Although most plant laboratories transform these model plants on a routine basis, protocols from leading experts may further enhance their capabilities. Parts III through VI in Volume 1 and Parts I through

VII in Volume 2 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. Part VIII in Volume 2 (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

Contents

Dedication	v
Preface	vii
Contributors	xiii
Contents for Volume 2	xix

PART I AGROBACTERIUM HANDLING

1 Culture and Maintenance of <i>Agrobacterium</i> Strains Arlene A. Wise, Zhenying Liu, and Andrew N. Binns	3
2 Binary Vectors and Super-binary Vectors Toshihiko Komari, Yoshimitsu Takakura, Jun Ueki, Norio Kato, Yuji Ishida, and Yukoh Hiei	15
3 Three Methods for the Introduction of Foreign DNA into <i>Agrobacterium</i> Arlene A. Wise, Zhenying Liu, and Andrew N. Binns	43
4 Integration of Genes into the Chromosome of <i>Agrobacterium</i> <i>tumefaciens</i> C58 Lan-Ying Lee	55
5 Nucleic Acid Extraction from <i>Agrobacterium</i> Strains Arlene A. Wise, Zhenying Liu, and Andrew N. Binns	67
6 <i>Agrobacterium</i> Virulence Gene Induction Stanton B. Gelvin	77

PART II MODEL PLANTS

7 <i>Arabidopsis thaliana</i> Floral Dip Transformation Method Andrew Bent	87
8 <i>Agrobacterium</i> Transformation of <i>Arabidopsis thaliana</i> Roots: A Quantitative Assay Stanton B. Gelvin	105
9 <i>Medicago truncatula</i> Transformation Using Leaf Explants Viviane Cosson, Patricia Durand, Isabelle d'Erfurth, Adam Kondorosi, and Pascal Ratet	115
10 <i>Medicago truncatula</i> Transformation Using Cotyledon Explants Elane Wright, Richard A. Dixon, and Zeng-Yu Wang	129
11 <i>Medicago truncatula</i> Transformation Using Root Explants Cynthia Crane, Richard A. Dixon, and Zeng-Yu Wang	137

- 12 *Nicotiana* (*Nicotiana tobaccum*, *Nicotiana benthamiana*)
Tom Clemente 143
- 13 Generation of Composite Plants Using *Agrobacterium rhizogenes*
Christopher G. Taylor, Beth Fuchs, Ray Collier,
and W. Kevin Lutke 155

PART III CEREAL CROPS

- 14 Barley (*Hordeum vulgare* L.)
John Jacobsen, Ingrid Venables, Ming-Bo Wang,
Peter Matthews, Michael Ayliffe, and Frank Gubler 171
- 15 Maize (*Zea mays* L.)
Bronwyn R. Frame, Tina Paque, and Kan Wang 185
- 16 Indica Rice (*Oryza sativa*, BR29 and IR64)
Karabi Datta and Swapan Kumar Datta 201
- 17 Japonica Rice Varieties (*Oryza sativa*, Nipponbare, and Others)
Philippe Hervé and Toshiaki Kayano 213
- 18 Rye (*Secale cereale* L.)
Fredy Altpeter 223
- 19 Sorghum (*Sorghum bicolor* L.)
Zuo-yu Zhao 233
- 20 Wheat (*Triticum aestivum* L.)
Yuechun Wan and Jeanne Layton 245

PART IV INDUSTRIAL PLANTS

- 21 Canola (*Brassica napus* L.)
Vinitha Cardoza and C. Neal Stewart, Jr. 257
- 22 Cotton (*Gossypium hirsutum* L.)
Keerti S. Rathore, Ganesan Sunilkumar,
and LeAnne M. Campbell 267
- 23 Indian Mustard [*Brassica juncea* (L.) Czern.]
Ksenija Gasic and Schuyler S. Korban 281
- 24 Sunflower (*Helianthus annuus* L.)
Dalia M. Lewi, H. Esteban Hopp, and Alejandro S. Escandón 291

PART V LEGUME PLANTS

- 25 Alfalfa (*Medicago sativa* L.)
Deborah A. Samac and Sandra Austin-Phillips 301
- 26 Chickpea (*Cicer arietinum* L.)
Kiran Kumar Sharma, Pooja Bhatnagar-Mathur,
and Boddu Jayanand 313

27	Clovers (<i>Trifolium</i> spp.) Aidyn Mouradov, Stephen Panter, Marcel Labandera, Emma Ludlow, Michael Emmerling, and German Spangenberg ..	325
28	Peas (<i>Pisum sativum</i> L.) Jan Grant and Pauline Cooper	337
29	Peanut (<i>Arachis hypogaea</i> L.) Kiran Kumar Sharma and Pooja Bhatnagar-Mathur	347
30	Pigeonpea (<i>Cajanus cajan</i> L. Millsp.) Kiran Kumar Sharma, Gopinath Sreelatha, and Sunitha Dayal	359
31	Red Clover (<i>Trifolium pratense</i>) Michael L. Sullivan and Kenneth H. Quesenberry	369
32	Soybean (<i>Glycine max</i>) Transformation Using Mature Cotyledonary Node Explants Paula M. Olhoft, Christopher M. Donovan, and David A. Somers	385
33	Soybean (<i>Glycine max</i>) Transformation Using Immature Cotyledon Explants Tae-Seok Ko, Schuyler S. Korban, and David A. Somers	397
34	Tepary Bean (<i>Phaseolus acutifolius</i>) Mukund Zambre, Marc Van Montagu, Geert Angenon, and Nancy Terryn	407
PART VI VEGETABLE PLANTS		
35	<i>Brassica oleracea</i> Penny A. C. Sparrow, Philip J. Dale, and Judith A. Irwin	417
36	Cucumber (<i>Cucumis sativus</i> L.) Wojciech Burza, Sabina Zuzga, Zhimin Yin, and Stefan Malepszy	427
37	Eggplant (<i>Solanum melongena</i> L.) Joyce Van Eck and Ada Snyder	439
38	Lettuce (<i>Lactuca sativa</i> L.) Ian S. Curtis	449
39	Tomato (<i>Lycopersicum esculentum</i>) Joyce Van Eck, Dwayne D. Kirk, and Amanda M. Walmsley	459
Index		475

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Contents for Volume 2

Preface

Contributors

Contents for Volume 1

PART I ROOT PLANTS

- 1 Carrot (*Daucus carota* L.)
Owen Wally, Jayaraj Jayaraman and Zamir K. Punja
- 2 Cassava (*Manihot esculenta* Crantz)
**Weston Msikita, Uzoma Ihemere, Dimuth Siritunga,
and Richard T. Sayre**
- 3 Potato (*Solanum tuberosum* L.)
Steve Millam
- 4 Sweet Potato [*Ipomoea batatas* (L.) Lam.]
Guo-qing Song and Ken-ichi Yamaguchi

PART II TURF GRASSES

- 5 Bermudagrass (*Cynodon* spp.)
Yaxin Ge and Zeng-Yu Wang
- 6 Perennial Ryegrass (*Lolium perenne* L.)
Fredy Altpeter
- 7 Switchgrass (*Panicum virgatum* L.)
Mariya N. Somleva
- 8 Tall Fescue (*Festuca arundinacea* Schreb.)
Yaxin Ge and Zeng-Yu Wang
- 9 Turf Grasses
**Chip Longo, Colin Lickwar, Qian Hu, Kimberly Nelson-Vasilchik,
David Viola, Joel Hague, Joel M. Chandlee, Hong Luo,
Albert P. Kausch**

PART III WOODY SPECIES

- 10 American Elm (*Ulmus americana*)
**Andrew E. Newhouse, Franziska Schrodtt, Charles A. Maynard,
William A. Powell**
- 11 Cork Oak Trees (*Quercus suber* L.)
**Rubén Álvarez, Mariano Toribio, Millán Cortizo,
and Ricardo-Javier Ordás Fernández**

- 12 Eucalyptus
**Zenn-Zong Chen, Cheng-Kuen Ho, In-Suk Ahn,
and Vincent L. Chiang**
- 13 Pine (*Pinus radiata*)
Jan Grant, Tracy Dale and Pauline Cooper
- 14 Poplar (*Populus* spp.)
Richard Meilan and Caiping Ma
- 15 Rubber Tree (*Hevea brasiliensis* Muell. Arg)
**Perumal Venkatachalam, Radha Jayashree, Karumamkandathil Rekha,
Sreedharannair Sushmakumari, Sankaren Sobha,
Parukkuttyamma Kumari Jayasree,
Radha Gopikkuttanunithan Kala, and Arjunan Thulaseedharan**

PART IV TROPIC PLANTS

- 16 Banana (*Musa* sp.)
**Juan B. Pérez Hernández, Serge Remy, Rony Swennen,
and László Sági**
- 17 Citrus
Vladimir Orbović and Jude W. Grosser
- 18 Coffee (*Coffea* sp.)
**Thierry Leroy, Edgardo Alpizar, Magali Dufour,
and Hervé Etienne**
- 19 Papaya (*Carica papaya* L.)
Yun J. Zhu, Maureen M. M. Fitch, and Paul H. Moore
- 20 Pineapple [*Ananas comosus* (L.) Merr.]
**Lourdes Yabor, Patricia Espinosa, Ariel D. Arencibia,
and José C. Lorenzo**
- 21 Sugarcane (*Saccharum* spp.)
Ariel D. Arencibia and Elva R. Carmona

PART V NUTS AND FRUITS

- 22 American Chestnut [*Castanea dentata* (Marsh.) Borkh.]
**Charles A. Maynard, Linda D. Polin, Sharon LaPierre,
Ronald E. Rothrock, and William A. Powell**
- 23 Apple (*Malus* × *domestica*)
**Abhaya M. Dandekar, Gianni Teo, Sandra L. Uratsu,
and David Tricoli**
- 24 Blueberry (*Vaccinium corymbosum* L.)
Guo-Qing Song and Kenneth C. Sink

- 25 Grapevine (*Vitis vinifera* L.)
**Alain Bouquet, Laurent Torregrosa, Pat Iocco,
and Mark R. Thomas**
- 26 Strawberry (*Fragaria* × *ananassa*)
Bruno Mezzetti and Elisa Costantini
- 27 Walnut (*Juglans*)
**Chuck Leslie, Sandra L. Uratsu, Gale McGranahan,
and Abhaya M. Dandekar**

PART VI ORNAMENTAL PLANTS

- 28 Carnation (*Dianthus caryophyllus* L.)
Chalerm Sri Nontaswatsri and Seiichi Fukai
- 29 Chrysanthemum (*Dendranthema* × *grandiflora*)
Jaime A. Teixeira da Silva
- 30 Orchids (*Cymbidium* spp., *Oncidium*, and *Phalaenopsis*)
Ming-Tsair Chan, Yuan-Li Chan and Sanjaya
- 31 Petunia (*Petunia hybrida*)
W. Kevin Lutke
- 32 Rose (*Rosa hybrida* L.)
Schuyler S. Korban, Ksenija Gasic, and Xiangqian Li

PART VII MEDICINAL PLANTS

- 33 Ginseng (*Panax ginseng*)
Yong Eui Choi
- 34 Hemp (*Cannabis sativa* L.)
Mistianne Feeney and Zamir K. Punja
- 35 Opium Poppy (*Papaver somniferum*)
Julie A. Chitty, Robert S. Allen, and Philip J. Larkin

PART VIII NON-PLANTS

- 36 Actinomycetes (*Streptomyces lividans*)
Clarence I. Kado and Brian Kelly
- 37 Filamentous Fungi (*Magnaporthe grisea* and *Fusarium oxysporum*)
**Chang Hyun Khang, Sook-Young Park, Hee-Sool Rho,
Yong-Hwan Lee, and Seogchan Kang**
- 38 Green Alga (*Chlamydomonas reinhardtii*)
Manchikatla V. Rajam and S. Vinod Kumar
- 39 Mammalian Cells
Tzvi Tzfira, Talya Kunik, Yedidya Gafni, and Vitaly Citovsky

- 40 Mushroom (*Agaricus bisporus*)
C. Peter Romaine and Carl Schlagnhauer
- 41 Yeast (*Saccharomyces cerevisiae*)
**Paul J. J. Hooykaas, Amke den Dulk-Ras, Paul Bundock,
Jalal Soltani, Haico van Attikum, and G. Paul van Heusden**

Index

I

***AGROBACTERIUM* HANDLING**

Culture and Maintenance of *Agrobacterium* Strains

Arlene A. Wise, Zhenying Liu, and Andrew N. Binns

Summary

As aerobic chemoorganotrophs, most *Agrobacterium* strains will grow on a wide range of complex and defined media. Methods commonly used for the culture and storage of other chemoorganotrophs will usually work for agrobacteria as well. Problems with culture or strain maintenance will occur more frequently because of careless technique than because of strain difficulties. Here we describe a few of the complex and defined media that have been successfully used in the growth of agrobacteria including some that are semiselective for agrobacteria. Finally, we present methods suitable for short- and long-term storage of *Agrobacterium* strains.

Key Words: Complex media; defined media; minimal media; selective media; enrichment media; stab cultures; dessication; vermiculite.

1. Introduction

Most strains of *Agrobacterium tumefaciens* and its avirulent cousin *A. radiobacter* are able to grow on minimal media with salts and a simple carbon source. *A. rhizogenes*, *A. rubi*, and some other natural isolates are auxotrophs that require the addition of growth factors such as biotin, nicotinic acid, pantothenate, and (or) glutamate to minimal medium. Carbon sources that are not readily used, even by the more nutritionally proficient members of the genus, include cellulose and starch. Nitrate or ammonium salts are sufficient nitrogen sources for *A. tumefaciens* and *A. radiobacter*, but not for *A. rubi* or *A. rhizogenes* (1–3).

There are many methods for storage of bacteria, and most will be suitable for maintenance of *Agrobacterium*. Important considerations in choosing a storage method include the length of time cells can be expected to remain viable, the genetic stability of the stored population, the number and value of the cultures to be maintained, and the frequency with which access to the cultures will be required (4). Here, we describe three methods suitable for

short-term (3 mo to a year) or long-term (indefinite) storage of *Agrobacterium*. The methods described include maintenance on stab cultures, desiccation on vermiculite, and low-temperature freezing.

Stab cultures are a simple and inexpensive method for maintenance of *Agrobacterium* strains with transfer to fresh medium at regular intervals. If one has few cultures to maintain, it may be the best choice, but note that there is some risk of genetic change through mutant selection and plasmid loss. In addition, the potential for contamination rises with successive transfers.

An easy and inexpensive method of strain storage consists of desiccation in a protective environment. Pesenti-Barili et al. (5) found an 80% survival rate for *A. radiobacter* 13 mo after inoculation of sterile vermiculite and storage at 4°C. In that study, the author's intent was to identify a method for preservation of a biologically active strain appropriate for commercial purposes. We present a scaled-down version of their system as a suitable method for the short-term storage of *Agrobacterium* strains.

For laboratories with a large number of strains, the best long-term (indefinite) method of preservation is the freezing of small cultures at -70 or -80°C. Freezing a strain eliminates the possibility of genetic change, and the method requires no handling for maintenance purposes after the initial storage event. On the other hand, the cost of a low-temperature freezer is substantial, and arrangements will be needed to protect against failure of the freezer owing to electrical interruptions or mechanical breakdown.

2. Materials

2.1. Complex Media (see Notes 1–4)

1. Yeast-mannitol medium: 10 g/L mannitol, 1 g/L yeast extract, 0.5 g/L K_2HPO_4 , 0.2 g/L $CaCl_2$, 0.2 g/L NaCl, 0.2 g/L $MgSO_4 \cdot 7H_2O$, 10 mg/L $FeCl_3$.
 - a. Dissolve all ingredients in 900 mL of water.
 - b. Adjust pH to 7.0.
 - c. Bring the volume to 1 L.
 - d. For *A. rubi*, the addition of biotin, nicotinic acid, and calcium pantothenate, each at 200 µg/L, will improve growth. For *A. rhizogenes* and *A. vitis*, add biotin.
2. Nutrient-yeast medium: 8 g/L nutrient broth powder, 2 g/L yeast extract.
 - a. Dissolve ingredients in water.
 - b. Bring volume to 1 L and autoclave.
 - c. When making nutrient broth or agar, simply leave out the yeast extract.
3. YDPC medium: 4 g/L peptone, 4 g/L yeast extract, 5 g/L $(NH_4)_2SO_4$, 10 g/L $CaCO_3$, glucose (20% solution).
 - a. Dissolve first four ingredients in 900 mL water.
 - b. Bring the volume to 1 L.

- c. Check that pH is close to 7.0.
- d. Autoclave.
- e. Autoclave the glucose separately and add 100 ml/L to the cooled medium.
4. MG/L medium: 5 g/L tryptone, 2.5 g/L yeast extract, 5 g/L NaCl, 5 g/L mannitol, 0.1 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g/L K_2HPO_4 , 1.2 g/L L-glutamate, thiamine (10% solution, filter-sterilized).
 - a. Dissolve all ingredients except thiamine in 900 mL water.
 - b. Check that pH is close to 7.0.
 - c. Bring volume to 1 L.
 - d. Autoclave.
 - e. When media cools to 60°C or below, add 120 μL /L of the thiamine solution.
5. LB: 10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract.
 - a. Dissolve ingredients in water.
 - b. Bring volume to 1 L.
 - c. Check that pH is close to 7.
 - d. Autoclave.
6. YEB medium: 5 g/L tryptone, 1 g/L yeast extract, 5 g/L nutrient broth, 5 g/L sucrose, 0.49 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$.
 - a. Dissolve ingredients in water.
 - b. Adjust the pH to 7.2 and bring volume to 1 L.
 - c. Autoclave.

2.2. Defined Media: AB* (AB*I) Medium (see Notes 1, 2, 5, and 6)

1. (see Note 6): prepare and autoclave separately a 20X AB* salts solution (20 g/L NH_4Cl , 6 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3 g/L KCl, 0.2 g/L CaCl_2 , and 15 mg/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), a 500 mM phosphate solution (60 g/L K_2HPO_4 and 20 g/L NaH_2PO_4 , pH 7.5), an AB* buffer [3.9 g/L 2-(N-morpholino)ethane sulfonic acid (MES; pH to 5.5 with KOH)], and a 20% carbon source (glycerol, arabinose, glucose, or sucrose) solution.
2. Filter-sterilize a 10% solution of thiamine and (optional) a 10% solution of casamino acids.
3. Cool autoclaved solutions to room temperature.
4. For 1 L of medium, add 50 mL AB* salts and 2.4 mL of the phosphate solution to the MES buffer.
5. Add 100 μL thiamine.
6. Add the carbon source to 0.2%.
7. If using casamino acids, add them to 0.05%.
8. If you are preparing *vir* gene induction medium, add a phenolic inducer, e.g., 3,5-dimethoxy-4-hydroxy-acetophenone (acetosyringone) dissolved in dimethyl sulfoxide (DMSO). The amount of phenolic inducer will depend on your strain and the desired level of *vir* gene induction.

2.3. Enrichment and Selective Media

1. Medium 1A for *A. tumefaciens* and related strains (3; see Notes 1, 2, and 7): 3.04 g/L L-arabitol, 1.04 g/L K_2HPO_4 , 0.54 g/L KH_2PO_4 , 0.16 g/L NH_4NO_3 , 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.29 g/L sodium taurocholate, 2 ml/L 1% crystal violet, and 15 g/L agar.
 - a. Dissolve the first seven ingredients in the water.
 - b. Bring the volume to 1 L.
 - c. Add to the agar and autoclave.
 - d. Cool to 50°C and add 10 mL each of the following: cycloheximide (2% solution) and $Na_2SeO_3 \cdot 5H_2O$ (1% solution).
 - e. Optional: K_2TeO_3 (100 mg/mL stock in ultrapure water) can be added to 80 µg/mL to improve the selectivity of the medium for *Agrobacterium* strains (11).
2. Medium 1E for *A. rhizogenes* (3, see Notes 1, 2, and 7): 3.05 g/L erythritol, 1.04 g/L K_2HPO_4 , 0.54 g/L KH_2PO_4 , 0.16 g/L NH_4NO_3 , 0.25 g/L $MgSO_4 \cdot 7H_2O$, 0.29 g/L sodium taurochlorate, 1 ml/L 1% solution of yeast extract, 5 ml/L 0.1% solution malachite green, and 15 g/L agar.
 - a. Dissolve the first eight ingredients in water.
 - b. Bring volume to 1 L.
 - c. Add to the agar and autoclave.
 - d. Cool to 50°C.
 - e. Add 10 mL each of the following: cycloheximide (2% solution) and $Na_2SeO_3 \cdot 5H_2O$ (1% solution).
 - f. Optional: K_2TeO_3 (100 mg/mL stock in ultrapure water) can be added to 160 µg/mL to improve the selectivity of the medium for *Agrobacterium* strains (11).

2.4. Antibiotics for *Agrobacterium* Selection and Culture (see Table 1 and Note 8)

2.5. Strain Storage

1. Sterile vials with screw-cap tops, e.g., Corning, cat. no. 430489 (see Note 9).
2. Fresh plate or liquid culture of strain to be stored (see Note 10).
3. Protective solution: 5% sucrose in sterile skim milk (autoclaved).
4. Fine or extrafine vermiculite, expanded type (see Note 11).
5. Precision balance.
6. Oven.
7. Low-temperature freezer (see Note 12).
8. 50% Glycerol in MG/L, YEB, or other liquid medium (autoclaved).

3. Methods

3.1. Media Preparation

In general, the pH of media for the growth of *Agrobacterium* should be between pH 6.8 and 7.2. A noted exception is AB*I (*vir* gene induction)

Table 1
Antibiotics Used in the Culture
and Selection of Resistant *Agrobacterium* Strains^a

Antibiotic	Stock concentration (mg/mL)	Solid medium (μg/mL)	Liquid medium (μg/mL)
Carbenicillin	100 in water	100	30–50
Chloramphenicol ^b	3 in ethanol	3	3
Erythromycin	100 in ethanol	150	100
Gentamicin	100 in water	100	100
Kanamycin	50 in water	50	10–20
Rifampicin	10 in methanol	10	10
Spectinomycin	100 in water	100	25–50
Tetracycline ^b	3 in 50% ethanol	3	1.5

^aSee **Note 8**. Note that antibiotic concentrations may need to be adjusted depending on the strain and the copy number of the gene determining resistance.

^bSome *Agrobacterium* strains have natural resistance to chloramphenicol and tetracycline.

medium, in which the acidic pH helps maximize *vir* gene induction (10). Liquid medium can be converted to plate media by adding it to 15 g/L agar before autoclaving. Polystyrene Petri dishes (100 × 15 mm) are suitable for most plate media and are available from several biological supply companies, including Fisher Scientific.

The ideal growth temperature for *Agrobacterium* is 25–30°C. Repeated subculture or growth at high temperature (e.g., 37°C) risks loss of the megaplasmid that determines virulence (1,14). Depending on the strain and the composition of plate medium, it will take 2–4 days for colonies to appear at 25°C. The growth of even prototrophic strains of *Agrobacterium* will proceed faster when amino acids and vitamins are supplied either as a component of complex media or with supplementation of defined media. Another determinant of growth rate is the “age” of the inoculum. This is most apparent with liquid cultures: the inoculation of media with cells from a smaller overnight culture still in the exponential or early stationary phase of growth will shorten the lag phase that precedes exponential growth in the larger culture. For large liquid cultures, good aeration may be achieved by shaking (at 220 rpm) growing cells in Erlenmeyer flasks with a volume four or five times that of the culture, e.g., 200 mL in a 1-L flask. For smaller cultures (e.g., 2–3 mL), autoclavable glass tubes (16 × 125 mm) are available from Fisher Scientific.

3.2. Strain Storage

3.2.1. Storage of *Agrobacterium* Strains through Stab Cultures

1. Prepare 100 mL of sterile nutrient agar (see **Note 13**). Cool the medium to between 50 and 60°C for ease of handling.
2. Aseptically transfer 1 to 2 mL of the medium to each sterile tube. Loosely cap the tubes. When the agar sets, tighten caps and cover the tubes with foil or plastic wrap to prevent spores and fungi from settling on the tubes. Stab medium can be stored for several months at 4°C.
3. To inoculate a stab culture, use a flame-sterilized loop to pick up a single colony of *Agrobacterium* cells and stab it into the center of the medium. Repeat for one or more backup cultures. Immediately cap the tube tightly, and seal it with parafilm.
4. Incubate the inoculated stab culture tube at 25°C for 2 d.
5. Stab cultures can be stored at room temperature or (best) at 4°C for 4–6 mo.
6. Transfer the strain to new stabs every few months after streaking for single colonies and confirming genetic characteristics with antibiotic resistance, polymerase chain reaction, and/or virulence tests (see **Note 14**).
7. To access the stored culture, use a sterile inoculating loop to remove a small amount of the culture to a nutrient agar plate. Streak to obtain independent colonies.

3.2.2. Storage of *Agrobacterium* Strains through Desiccation

1. Begin preparation for strain storage by washing the vermiculite with distilled water. Drain it well, and place it in a warm (or very warm) oven 80 to 150°C (see **Note 15**). Periodically, check the weight of the vermiculite. When its weight stabilizes, the vermiculite is dry.
2. Place 0.2 g of vermiculite into several 2-mL screw-cap tubes. Autoclave the tubes with vermiculite for 30 min (see **Note 16**).
3. Dry vermiculite in tubes in the oven 80–150°C until dry, as indicated by stable weight.
4. Prepare overnight culture(s) of strains to be stored in nutrient broth with any antibiotics necessary for plasmid maintenance.
5. Use the overnight culture(s) to inoculate separate flasks containing 150 mL YDPC with the appropriate antibiotics. Grow to early or middle stationary phase (see **Notes 17 and 18**).
6. Pellet the cells by centrifugation. Remove the supernatant. Resuspend the cells from 150 mL of YDPC in 0.8 to 1.0 mL protective solution. Use a 200-μL aliquot of the cells to inoculate each tube of vermiculite. Vortex or shake briefly to distribute the culture through the vermiculite (see **Note 19**). Incubation is not required.
7. The vermiculite culture can be stored in room temperature for 4–6 mo, but longevity is increased by storage at 4°C (see **Notes 14 and 20**).

8. To access the culture, aseptically place a bit of the vermiculite into a microcentrifuge tube containing 100 μL sterile 0.8% NaCl. Vortex well to wash the bacteria into the saline. Remove about 25 μL of the saline to an MG/L (or other) agar plate with appropriate antibiotics and streak for single colonies.

3.2.3. Storage of *Agrobacterium* Strains by Low-Temperature Freezing

1. Inoculate 2 or 3 mL of media (MG/L or YEB are a good choices) that contains the appropriate antibiotics for plasmid maintenance with your strain (see **Note 21**).
2. Grow the culture to early or middle stationary phase (usually overnight). Chill the culture and the sterile medium containing 50% glycerol on ice.
3. Add an equal volume of the medium with glycerol to make the culture 25% glycerol. Mix culture and medium completely.
4. Transfer the cells to a labeled 2-mL tube, and place it in the freezer (see **Notes 12** and **14**). The frozen culture can be stored indefinitely.
5. To recover the strain, simply scrape a bit of the frozen culture onto plate medium containing the appropriate antibiotics. This can be done with a sterile, cooled, inoculating loop or with a sterile pipet tip. It is important to handle the culture quickly to prevent thawing and refreezing. If handled properly, the same culture can be used repeatedly for retrieving the bacteria strain.

4. Notes

1. Media ingredients are available from a number of biological supply companies including Fisher Scientific, BD-BBL/Difco, and Sigma-Aldrich.
2. Autoclave-generated steam at a temperature of 121°C and pressure near 15 lb/in² is the most common method of sterilizing bacterial growth media. Recommended autoclave times range from 15 min to 1 h. It should be noted that agar conducts heat poorly. The temperature in the center of 500 mL of a 1.2% agar solution was found to reach 121°C 40 min after the internal chamber of the autoclave reached that temperature (6). Thus, even a 30-min autoclave time would be insufficient to kill heat-resistant microbial spores. If the growth of fungi and spore-forming organisms on autoclaved medium is a problem, increase the surface area of the media. One may do this by changing the size and shape of the container or by dividing the media or solutions into multiple containers.
3. Media sterilization through autoclaving requires consideration of the effect of heat on certain nutrients. Sucrose and other glycosides with furanoside groups will hydrolyze when heated at acid pH (6). The heating of reducing sugars, e.g., glucose, and/or phosphate, and/or amino acids or peptides together, may create toxic compounds or make nutrients unavailable (7). The reaction of sugar with other media components becomes a problem when concentrated solutions are heated at alkaline pH. Thus, sugars and peptones should be dissolved (not lie together at the bottom of the autoclave container). Media consisting of a sugar-peptide solution should not be prepared in concentrated form, and pH should generally be less than 8.0 (7). Alternatively, one can autoclave (or filter-sterilize) sugar solutions separately for addition to other media components after autoclaving.

4. All *Agrobacterium* strains should be able to grow on complex media. A survey of the literature indicates that *Agrobacterium* is frequently grown in Luria-Bertani (LB), nutrient broth (8 g/l), with or without the addition of yeast extract or glucose, and many other complex media. Potato dextrose agar plates are a common medium for the growth of *Agrobacterium*. These media are commercially available, and their formulas are readily available in microbiology handbooks and by Internet searches.
5. Putting together a defined medium requires the separate sterilization of its various components. For example, magnesium, potassium, ammonium, sodium, and phosphate ions can become unavailable when heated together owing to the formation of various insoluble magnesium phosphate salts. Thus, the magnesium source should be prepared and autoclaved separately from the phosphate source (6). Also, note that an acidic solution of sucrose is subject to hydrolysis when heated (6). This is of particular importance when preparing media for analysis of *A. tumefaciens* virulence genes, as glucose, but not sucrose will act as a stimulant of *vir* gene induction (8).
6. AB* medium is a derivative of AB medium (9). With the addition of a phenolic inducer, AB* becomes AB*I (AB* induction medium) for use as an inducing medium for *A. tumefaciens* virulence genes. For expression of the virulence genes at high levels, choose arabinose or glucose as the carbon source (8) and limit the addition of AB* phosphates to 1.2 mM (10). Otherwise, phosphate can be added to 10 mM. Casamino acids will increase the growth rate of *Agrobacterium* in AB* medium, particularly when the carbon source is glycerol. The addition of casamino acids does not effect *vir* gene expression in *A. tumefaciens* as measured by a *virB-lacZ* fusion (our personal observation). If making AB*I agar plates, it is necessary to autoclave the agar separately. Heating agar in acidic medium prevents it from becoming sufficiently solid upon cooling. Prepare the MES buffer solution as a threefold concentrate, i.e., 3.9 g MES in 300 mL water, and adjust the pH to 5.5. Autoclave 15 g agar in enough water (approximately 600 mL) to make the total volume 1 L after the additions of salts, phosphate, carbon source, and so on. The acidity (pH 5.5) of AB*I medium increases expression of the *vir* genes (10). However, the medium can also be made with pH near 7.0 by buffering with 25 mM Bis-Tris rather than MES when a nonacidic defined medium is desired.
7. The selective nature of enrichment media depends primarily on the presence of substances that are toxic to fungi and some bacteria. *Agrobacterium* forms shiny, white, raised colonies that may become mucoid or turn orange-brown after extended incubation on media 1A and 1E (3). The addition of tellurite at 80 µg/mL for 1A medium and at 160 µg/mL for 1E medium improves selectivity for *Agrobacterium*, although *Rhodobacter* and other *Rhizobium* species are also tellurite resistant (11). *Agrobacterium* will form shiny, convex, black colonies on medium amended with tellurite. The identity of selected strains should be confirmed with 16s RNA analysis and probes specific to pTi or pRi (11).

8. In general, antibiotic stock solutions are filter-sterilized and stored at -20°C . Antibiotics should be added to medium at temperatures below 60°C . Plate media that contain antibiotics should be labeled with the date of preparation and stored at 4°C . For liquid media, add the antibiotic just before inoculating the culture. Note that the half-life of many antibiotics in plate media is relatively short. If in doubt as to the efficacy of the antibiotic, test the plate with a strain that you know to be sensitive to the antibiotic. Ampicillin cannot be used for selection of *Agrobacterium* carrying a plasmid with the β -lactamase (*bla*) gene in rich medium. Carbenicillin may be used instead. *A. tumefaciens* C58 contains *tetA* and *tetR* genes similar to the Tet(A) class of determinants of tetracycline resistance (12). A high concentration of tetracycline induces tetracycline resistance in *A. tumefaciens* C58 and hinders identification of colonies that are tetracycline resistant owing to plasmid transformation. At concentrations of tetracycline below $5\text{ }\mu\text{g/mL}$, the number of spontaneously resistant colonies is reduced or eliminated. *A. tumefaciens* strains C58, A136, and BG53 are chloramphenicol resistant owing to the presence of the *catB* gene (13). *A. tumefaciens* LBA4404 and GV3101 are chloramphenicol-sensitive strains.
9. The choice of tubes and labels will depend on the method chosen for storage. It is convenient to use small (2-mL) tubes. Tubes may be plastic or glass with caps that fit snugly to limit air exchange and prevent contamination. It is convenient for the tubes to have a surface designed for ease of labeling with a pen. However, depending on the storage method, tape will provide an adequate label. For strains to be stored frozen, note that tape may fall off after a few years.
10. Cultures of strains to be stored should be inoculated from healthy single colonies or a fresh liquid culture grown in the same medium to be used in the preservation step.
11. Vermiculite can be purchased at nurseries or garden centers. Vermiculite sold in the United States has been tested and confirmed to be free of asbestos.
12. Temperatures for freezing bacteria should be below -30°C owing to uneven freezing of eutectic mixtures (4). Storage at temperatures between -65 and -80°C is common and gives good results for very long (indefinite) periods of storage. It is not necessary to fast-freeze with dry ice and ethanol or liquid nitrogen.
13. Storage of stab cultures (like other methods) depends, in part, on reducing the cell's metabolic rate. *Agrobacterium* will survive at room temperature for many months on stabs made with nutrient agar. Yeast-manitol agar is another good choice for stab culture media. Longest survival times occur when storage of inoculated stabs is at 2 to 4°C (3,4).
14. Clear labels and good records are important components of all storage methods. Labels should note the strain name and any plasmids it contains. Records and labels should be numerically coordinated so that someone can easily find the strain and information regarding its source, genetic characteristics, and antibiotic resistances.
15. Drying the vermiculite after washing and after autoclaving is necessary to control the proportion of the vermiculite carrier to moisture during storage of the bacteria.

16. Extra tubes can be prepared with vermiculite, autoclaved, and kept at room temperature for later use. Pesenti-Berili et al. (6) found that a second autoclaving of tubes and vermiculite 24 h after the first autoclaving eliminated the small amount of contamination found in an earlier trial of the method.
17. The amounts of nutrient broth and YPDC given here are for storage of one strain with backups.
18. Stationary phase cells develop resistance to stress that exponential cells may not have. However, if your cells grow too long after stationary phase, they will be in “death phase” and begin to die off. For cells grown in YDPC, try for an OD₆₀₀ between 1.8 and 2.0. Depending on the inoculum and your strain, this may take up to 2 d. A growth curve (OD₆₀₀ vs time) is useful to predict the length of time it takes for a specific strain to reach stationary phase in any particular media.
19. The vermiculite will still seem very dry after inoculation. Tight capping of the tube will allow moisture to be distributed throughout the vermiculite gradually, desiccating the bacteria.
20. The protective effects of vermiculite are believed to be owing to its ability to buffer pH shifts and absorb inhibitory metabolites. At room temperature, storage time will be on the order of 6 or more months, whereas it is reasonable to expect viable cells after more than 1 yr of storage at 4°C. It is prudent to perform a viability count occasionally to obtain an indication of when it is wise to reestablish the stored strains.
21. Prepare one or two backup cultures for storage. If feasible, store them in different freezers (make an arrangement with a colleague) to provide security against freezer failure.

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Binary Vectors and Super-binary Vectors

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Summary

A binary vector is a standard tool in the transformation of higher plants mediated by *Agrobacterium tumefaciens*. It is composed of the borders of T-DNA, multiple cloning sites, replication functions for *Escherichia coli* and *A. tumefaciens*, selectable marker genes, reporter genes, and other accessory elements that can improve the efficiency of and/or give further capability to the system. A super-binary vector carries additional virulence genes from a Ti plasmid, and exhibits very high frequency of transformation, which is valuable for recalcitrant plants such as cereals. A number of useful vectors are widely circulated. Whereas vectors with compatible selectable markers and convenient cloning sites are usually the top criteria when inserting gene fragments shorter than 15 kb, the capability of maintaining a large DNA piece is more important for consideration when introducing DNA fragments larger than 15 kb. Because no vector is perfect for every project, it is recommended that modification or construction of vectors should be made according to the objective of the experiments. Existing vectors serve as good sources of components.

Key Words: *Agrobacterium tumefaciens*; transformation; binary vector; super-binary vector.

1. Introduction

Research projects that involve transformation of higher plants are lengthy, complicated processes, which may last for years. The first parts of the projects are the steps of vector construction, performed by molecular biologists. Compared with the entire durations of the projects, these steps are relatively short and usually can be completed within weeks. However, scientists could make a series of irrevocable decisions and sometimes mistakes in these fate-determining steps, and the consequences would not emerge until years later.

Researchers often find undesired aspects of vector configuration when characterizing progeny of transgenic plants; in a worst case scenario these vector defects could ruin the entire project. In this chapter, steps from designing of vectors through preparation of strains of *Agrobacterium tumefaciens* ready for infection are addressed. It should be emphasized that good management of these processes can save a lot of precious time and resources.

Basic frameworks of the current vectors for transformation of higher plants were developed in the early and mid-1980s, soon after it had been elucidated that crown gall tumorigenesis represented the genetic transformation of plant cells (1). The first achievement was the removal of wild-type T-DNA, which causes tumors and inhibits plant regeneration, from Ti plasmids to generate “disarmed strains” such as LBA4404 (2). Earlier attempts at the introduction of engineered T-DNA into *A. tumefaciens* involved the placement of genes in *E. coli* vectors that could be integrated into a disarmed Ti plasmid (1). This was a reasonably efficient system, but a limitation was that the final product is a plasmid larger than 150 kb in *A. tumefaciens*, and confirmation of the structure was not straightforward.

Then the binary vector system was invented, exploiting the fact that the process for transfer of T-DNA is active even if the virulence genes and the T-DNA are located on separate replicons in an *A. tumefaciens* cell (2). An artificial T-DNA is constructed within a plasmid that can be replicated in both *A. tumefaciens* and *E. coli*. Plasmid construction is completed in *E. coli*, and simple transfer of the vector to *A. tumefaciens* produces a strain ready for plant transformation. Soon such binary vectors were widely distributed among plant scientists. Although the term *binary vector* literally refers to the entire system that consists of two replicons, one for the T-DNA and the other for the virulence genes, the plasmid that carries the T-DNA is frequently called a binary vector. We follow this popular and convenient terminology in this chapter.

One of the approaches toward enhancing the frequency of transformation by binary vectors is to employ additional virulence genes, such as *virB*, *virE*, and *virG*, which exhibit certain gene dosage effects (3–6). In the super-binary vector system, a DNA fragment that contains *virB*, *virC*, and *virG* from pTiBo542 is introduced into a small T-DNA-carrying plasmid (7). *A. tumefaciens* strains that carry pTiBo542 are wider in host range and higher in transformation efficiency than strains that carry other Ti plasmids, such as pTiA6 and pTiT37 (8). Super-binary vectors are highly efficient in the transformation of various plants (see **Note 1**) and played an important role when the host range of transformation mediated by *A. tumefaciens* was extended to important cereals in the mid-1990s (9,10). The final step of construction of a super-binary vector is integration of an intermediate vector with an acceptor vector in *A. tumefaciens*,

but, unlike the aforementioned integration system, the final product in the super-binary vector system is a plasmid that can be confirmed by routine restriction analysis of mini-scale DNA preparation from *A. tumefaciens*.

Commonly used binary and super-binary vectors are listed in **Table 1**. Helpful guidance for selection of the vectors has already been provided in the literature (**11**). Still, which is the best vector is a question with no definitive answer. Since these are vehicles for delivery of transgenes to plants, they should be (1) easy for the researcher to insert genes (loading), (2) efficient in plant transformation (unloading), (3) widely available to researchers, and (4) versatile for diverse purposes. Because such vectors can also be source materials for new vectors, if any vector components can be easily replaced or removed, the vector will be very useful. Recently constructed vectors provide a number of user-friendly features related to transgene loading and unloading, such as wide selection of cloning sites, high copy numbers in *E. coli*, high cloning capacity, improved compatibility with strains of choice, wide pool of selectable markers for plants, and high frequency of plant transformation. However, our quick survey of some 130 recently published papers, in which transformation of higher plants mediated by *A. tumefaciens* was described, revealed that derivatives of a relatively old vector, pBin19 (**12**), such as pBI121 (**13**), pIG121Hm (**9**), and others, were still used in about 40% of these studies. One reason could be that these vectors were widely circulated at early stages of plant transformation, and accumulated data in the literature from their use has built a lot of confidence. Another reason might be a convenient feature of pBI121, namely, that one-step replacement of the β -glucuronidase (Gus) gene with another gene can quickly create an overexpression vector for the gene.

No matter how difficult the choice is, we have to make a decision. In our laboratories, the key criteria for delivery of transgene fragments smaller than 15 kb are (1) compatibility of selectable markers with the experiments and (2) availability of convenient cloning sites. For delivering DNA fragments larger than 15 kb, the top consideration is whether the large DNA fragments can be cloned efficiently to the vectors and maintained stably in *E. coli* and *A. tumefaciens*, because large DNA pieces in certain vectors, e.g., high-copy-number vectors, may sometimes cause low efficiency of transformation of bacteria or rearrangement of the inserts (**14**).

The scope of transformation experiments in higher plants is complex, covering topics such as overexpression, regulated expression, downregulation or shut-down of foreign or internal genes, expression of gene fusion, assays of promoters or other regulatory elements, complementation of mutations with genomic sequences, tests of new molecular tools, tests of novel tissue culture protocols, and so on, with ever growing complexity. Therefore, in regard to the versatility of the vector, it is futile to try to design a vector that can be

Table 1
Commonly Used Binary and Super-binary Vectors

Vector	Plant selection marker	Bacterial selection marker	Source of borders	Replication origin for <i>A. tumefaciens</i>	Replication origin for <i>E. coli</i>	Mobilization	Reference, GenBank accession number, Contact
pBin19	Kan	Kan	pTiT37	IncP	IncP	Yes	(12), U09365, michael.bevan@bbsrc.ac.uk
pBI121	Kan	Kan	pTiT37	IncP	IncP	Yes	(13), AF485783, www.cambia.org
pGreen series ^a	Kan, Hyg, Sul, Bar	Kan	pTiT37 (synthetic)	IncW	pUC	No	(27), www.pgreen.ac.uk
pCAMBIA series	Kan, Hyg	Cm, Kan	pTiC58	pVS1	ColE1	Yes	www.cambia.org
pPZP series	Kan, Gen	Cm, Sp	pTiT37	pVS1	ColE1	Yes	(50), maliga@waksman.rutgers.edu
pPCV001	Kan	Ap	RB: pTiC58 LB: Octopine ^b	IncP	ColE1	Yes	(54), koncz@mpiz-koeln.mpg.de
pGA482	Kan	Tc, Kan	pTiT37	IncP	ColE1	Yes	(55), genean@postech.ac.kr
pCLD04541	Kan	Tc, Kan	Octopine	IncP	IncP	Yes	(49), AF184978, hbz.tamu.edu and www.jic.bbsrc.ac.uk/staff/ian-bancroft/vectorspage.htm
pBIBAC series	Kan, Hyg	Kan	Octopine	pRi	F factor	Yes	(47), www.biotech.cornell.edu/BIBAC/BIBACHomePage.html
pYLTAC series	Hyg, Bar	Kan	Octopine	pRi	Phage P1	No	(48), www.kazusa.or.jp/en/plant/TAC/index.html
pSB11	None	Sp	pTiT37	None	ColE1	Yes	(45), AB027256, www.jti.co.jp/plantbiotech
pSB1	None	Tc	None	IncP	ColE1	Yes	(45), AB027255, www.jti.co.jp/plantbiotech

^aIndication of more than one marker genes for a series of vectors means availability of a vector with each one of the markers.

^bSee **Note 3**.

suitable for all purposes. It is a good idea to modify existing vectors or to build new ones from scratch for specific purposes as demands arise.

2. Materials

2.1. Components of a Binary Vector

The compositions of widely circulated binary vectors are similar, and many of the components have been used for more than 15 yr without much modification in plant transformation experiments. Therefore, the components listed in this section are considered quite reliable. It is useful to prepare series of unfinished plasmids that carry various combinations of these elements as materials for molecular construction beforehand (see **Note 2**).

2.1.1. On the T-DNA

1. T-DNA borders and their sequence contexts. These components are usually DNA fragments cloned from well-known Ti plasmids. Imperfect, direct repeats of 25 bases, the right border (RB) and left border (LB), are said to be the only essential *cis* elements for T-DNA transfer (**15**) but factors that enhance (*overdrive*) or attenuate T-DNA transfer have been identified near the RB (**16,17**) or the LB (**17**), respectively. Therefore, it may be safe to retain a few hundred bases of natural sequences adjacent to the T-DNA. Sources for the borders are also indicated in **Table 1**. Data for the border sequences are available in GenBank, accession numbers ATU237588 for pTiC58, and ATACH5 for pTi15955 (see **Note 3**).
2. Multiple cloning sites (MCS). Many of the vectors have the MCS derived from popular cloning vectors, such as pUC8/9, pUC18/19, and pBluescript, whereas others have unique sequences. The MCS from these standard vectors are convenient because gene components are usually cloned in such vectors beforehand. Some vectors have the *lacZ* unit from the standard vectors, and blue/white selection for insertion of fragments may be employed.
3. Selectable marker gene cassette for plant transformation (see **Note 4**).
 - a. Promoters. Selectable markers need to be expressed in calli, in cells from those plants that are being regenerated, or germinating embryos to facilitate plant transformation. Therefore, promoters for constitutive expression are preferred. Promoters used mainly for dicotyledonous plants include the 35S promoter from cauliflower mosaic virus (**18**) and promoters derived from Ti plasmids, such as nopaline synthase (Nos) (**19**), octopine synthase (Ocs), mannopine synthase (Mas), gene 1, gene 2, and gene 7 (**20**). Popular promoters for monocotyledonous plants include the 35S promoter and the promoters from the ubiquitin (Ubi) gene of maize (**21**) and the actin (Act) gene of rice (**22**). The choice of promoters that drive the selectable marker genes affects the efficiency of transformation. For example, the Ubi promoter gave a frequency of transformation much higher than that of the 35S promoter in cereals (**23**; unpublished results).
 - b. Selectable markers for plants. Marker genes used in binary and super-binary vectors are listed in **Table 2**. Depending on the plants to be transformed,

Table 2
Selectable Markers (S) and Reporter Genes (R) Employed in Binary and Super-binary Vectors

Common abbreviations	Protein	S or R	Used for		Selection pressure	Ref.
			Plants	Bacteria		
NptII, Aph 3' II, Kan	Neomycin phosphotransferase II Aminoglycoside 3' phosphotransferase II	S	Yes	Yes	Kanamycin, G418, paromomycin	(56)
Hpt, Hph, AphIV, Hyg	Hygromycin phosphotransferase Aminoglycoside phosphotransferase IV	S	Yes	Yes	Hygromycin	(57)
NptI, Kan	Aminoglycoside phosphotransferase I	S		Yes	Kanamycin	(14)
NptIII, Kan	Aminoglycoside phosphotransferase III	S		Yes	Kanamycin	(58)
Bar, Pat	Phosphinothricin acetyl transferase	S	Yes		Phosphinothricin (BASTA), bialaphos	(59)
PMI	Phosphomannose isomerase	S	Yes		Mannose as sole carbon source	(60)
Ble	Bleomycin binding protein	S	Yes		Bleomycin, phleomycin	(61)
Sul	Mutant dihydropteroate synthase	S	Yes		Sulfonamide	(62)
BSD	Blasticidin deaminase	S	Yes		Blasticidin S	(63)
Als	Mutant acetolactate synthase	S	Yes		Sulfonylurea, imidazolinone, bispribac-sodium	(64)
AHAS	Mutant acetohydroxy acid synthase	S	Yes		Chlorsulfuron	(65)
DHFR	Dihydroforate reductase	S	Yes		Methotrexate	(66)
Gen	Gentamycin acetyltransferase	S	Yes	Yes	Gentamycin	(67)
EPSP	5-Enolpyruvylshikimate-3-phosphate synthase	S	Yes		Glyphosate (Round-up)	(68)
Ipt	Isopentenyl transferase	S	Yes		Cytokinin free	(69)
AadA, SPT, Spec	Aminoglycoside-3"-adenyltransferase Aminoglycoside nucleotidyl transferase	S	Yes	Yes	Spectinomycin, streptomycin	(70)
CAT, Cm	Chloramphenicol acetyltransferase	S, R	Yes	Yes	Chloramphenicol	(71)
Bla, Amp, Carb	β -Lactamase	S		Yes	Ampicillin, carbenicillin	(14)
Tet, TetA, TC	Tetracycline efflux protein	S		Yes	Tetracycline	(14)
Cah	Cyanamide hydratase	S	Yes		Cyanamide	(72)

Tdc	Tryptophan decarboxylase	S	Yes	4-Methyl tryptophan	(73)
XylA	Xylose isomerase	S	Yes	D-Xylose as sole carbon source	(74)
hemL, GSA-AT	Mutant glutamate-1-semialdehyde aminotransferase	S	Yes	Gabaculine	(75)
TfdA, DPAM	2,4-Dichlorophenoxyacetate monooxygenase	S	Yes	2,4-D	(76)
Bxn	3,5-Dibromo-4-hydroxybenzoic acid nitrilase	S	Yes	Bromoxynil	(77)
Pflp	Ferredoxin-like-protein	S	Yes	<i>Erwinia carotovora</i>	(78)
PPO	Mutant protoporphyrinogen oxidase	S	Yes	Butafenacil (herbicide)	(79)
DOGR	2-Deoxyglucose-6-phosphate phosphatase	S	Yes	2-Deoxyglucose	(80)
Gus, UidA	β -Glucuronidase	R	Yes		(13)
Luc	Luciferase	R	Yes		(33)
GFP	Green fluorescent protein ^a	R	Yes		(32)
LacZ	β -Galactosidase	R	Yes	Yes	(81)
Nos	Nopaline synthase	R	Yes		(82)
R-nj	Anthocyanin	R	Yes		(83)
OxO	Oxalate oxidase	R	Yes		(84)

^aA number of useful, distinctive derivatives of GFP and fluorescent proteins with different characteristics are available and reviewed in the literature (85).

the choice of selectable markers greatly affects the efficiency of transformation, and restrictive/permissive concentrations of selective agents vary considerably among plant species and even among cultivars (see **Note 5**). Kanamycin resistance is good for many dicotyledons including tobacco, tomato, potato, and *Arabidopsis* (see **Note 6**). Hygromycin resistance (*hpt*) is very good for rice transformation (**9**), and the phosphinothricin resistance gene (*bar*) is efficient for maize and other cereals (**10,24**). We do not think the effects of selectable markers have been explored sufficiently, because many of them were tested in very limited species of plants. Therefore, we made a comprehensive list of marker genes in **Table 2**, hoping that some of the markers may be investigated further to improve transformation of certain plant species.

- c. 3' Signal (see **Note 7**): DNA fragments of a few hundred bases derived from the 3' ends of the CaMV 35S transcript and *Agrobacterium* Nos and other T-DNA genes are carried by many of the binary and super-binary vectors.

2.1.2. On the Vector Backbone

1. Bacterial selectable marker gene. Genes that can confer resistance to kanamycin, gentamycin, tetracycline, chloramphenicol, spectinomycin, and hygromycin are popular markers for bacterial selection for both *E. coli* and *A. tumefaciens* (**Table 2**). Care must be exercised as some bacterial strains without vector plasmids have certain intrinsic antibiotic resistance. Some selectable markers for plants, such as Nos-nptII and 35S-hpt, give fair levels of resistance to both *E. coli* and *A. tumefaciens* (see **Note 8**). If such a dual function gene is present in the T-DNA, bacterial selectable markers may be omitted from the vector backbone to simplify the vector construction.
2. Plasmid replication functions. Binary vectors need to be replicated both in *E. coli* and *A. tumefaciens*. Use of plasmid replication functions with a wide host range, such as the ones of plasmid incompatibility group P (IncP) or W (IncW) is a good option. IncP binary vectors carry the origin of vegetative replication (OriV) and the transacting replication functions (Trf) of IncP plasmids (**25**). The replication locus of IncW plasmids, such as pSa, consists of the origin of replication and RepA gene (**26**). pGreen vectors have only the origin of replication, and the RepA function is provided by another plasmid, pSoup, in *A. tumefaciens* (**27**). Alternatively, replication functions for *A. tumefaciens*, such as the ones for an Ri plasmid (**28**) or pVS1 (**29**), and for *E. coli*, such as the ones for the F factor, phage P1, ColE1, or P15A (**14**), may be combined. Types of replication functions determine copy numbers and stability of the plasmids in bacterial cells. The use of high-copy plasmids for cloning of fragments larger than 15 kb can sometimes result in complications like rearrangement of DNA. If this is a problem, a good choice is an IncP plasmid, such as pBI121, which is a low-copy-number (about five copies per cell) plasmid in both *E. coli* and *A. tumefaciens* (see **Note 9**).
3. Plasmid mobilization functions (see **Note 10**). The origin of transfer (OriT) of IncP plasmids (**25**) or the bom function of ColE1 plasmid (**14**) is carried by most

of the binary vectors. Plasmids with one of the sequences may be mobilized from *E. coli* to *A. tumefaciens* aided by a conjugal helper plasmid, pRK2013 or pRK2073 (30). pRK2073 is a derivative of pRK2013 and has an insertion of Tn7 in the kanamycin resistance gene of pRK2013. Either one works fine for most applications, but pRK2073 is recommended if the target plasmid carries kanamycin resistance, and pRK2013 is recommended if the target has spectinomycin resistance.

2.2. Components of a Super-binary Vector

2.2.1. Intermediate Vector

1. T-DNA. The same composition described for a binary vector applies (see **Subheading 2.1.1.**).
2. Plasmid replication. An intermediate vector has an origin of replication of ColE1 like pBR322 and is replicated in *E. coli* but not in *A. tumefaciens*.
3. Plasmid mobilization. An intermediate vector has the bom function of ColE1 near the replication origin and can be mobilized by pRK2013 or pRK2073.
4. Bacterial selection. An intermediate vector has a spectinomycin resistance gene derived from Tn7 (31).
5. Homology with an acceptor vector. An intermediate vector and an acceptor vector share the 2.7-kb fragment that contains the ori and bom of ColE1 and the cos site from phage lambda (14) (the Ori-Cos fragment).

2.2.2. Acceptor Vector

1. Plasmid replication. An acceptor vector is an IncP plasmid and also has the ori of ColE1.
2. Plasmid mobilization. An acceptor vector has both the bom function of ColE1 and OriT of IncP plasmids and can be mobilized aided by pRK2013 or pRK2073.
3. Bacterial selection. An acceptor vector has tetracycline resistance derived from pRK2.
4. Virulence genes. An acceptor vector has a 14.8-kb *KpnI* fragment (the super-vir fragment) from pTiBo542. This fragment contains *virB*, *virC*, and *virG* operons (see **Note 1**).
5. Homology with intermediate vectors (see **Subheading 2.2.1.**).
6. T-DNA with a plant-selectable marker gene. An acceptor vector optionally has a T-DNA to create a vector for cotransformation.

2.3. Reporter Gene Cassette

1. Promoters. It is convenient to have a reporter gene expressed in various tissues and organs, and so the promoter is often chosen from the same group of promoters that may be used for selectable marker genes, which include 35S, Ubi, Act, Nos, and other T-DNA promoters. In some of the vectors, the promoter for the selectable marker and the reporter is the same, but, generally speaking, avoidance of duplication of the same components is recommended.

2. Reporter genes. β -Glucuronidase (Gus) (13), green fluorescent protein (GFP) (32) and luciferase (Luc) (33) are the most popular reporter genes (Table 2). Background activities in the assays of these enzymes are generally very low in higher plants. Reporter genes can be linked to regulatory sequences and used to study functionality of these sequences. Because Gus and GFP are highly stable proteins in plant cells (34), the activity of these proteins may not immediately reflect small or quick changes in the level of the mRNA for these proteins in plant cells. If this is the case, Luc, whose half-life in plant cells is much shorter than those of Gus and GFP (34), may be a good choice. A reporter gene that has an intron in the coding sequence, such as the *intron-Gus* gene (35), is very useful because this gene is not expressed in *A. tumefaciens*. In addition, especially in monocotyledons, introns can enhance expression for some genes (36). Introns placed close to the N-terminal in the coding sequence and in the 5' untranslated region of a gene may be equally effective (37).
3. 3' Signal. The 3' signal for a reporter gene may be chosen from the components listed for the selectable marker genes (see Subheading 2.1.1.).

2.4. Accessory Components for Binary and Super-binary Vectors

1. Restriction sites for endonucleases with long recognition sequences. Because genes have various restriction sites, it may not always be easy to find unique sites for introducing DNA sequences to a desired location on a vector. More than 10 restriction enzymes that recognize 8 bases are available now, and there are several homing endonucleases, which have recognition sites longer than 10 bases. It is useful to design vectors with a number of sites for these enzymes. Such vectors are especially useful for stacking of multiple expression units in one vector. Well-designed sets of plasmids that consists of a binary vector with these sites and high-copy cloning vectors with expression cassettes and subsets of the sites are called as *modular vectors* (38).
2. The sites for the GATEWAY® system. Molecular cloning based on restriction enzymes and DNA ligases is not always straightforward. The GATEWAY system (Invitrogen) is a cloning technology based on the site-specific recombination system of phage lambda. A step of molecular cloning may be performed in a single tube within a few hours, and *E. coli* that carries a desired plasmid is recovered at a very high frequency on the following day. In essence, a DNA fragment flanked by a pair of short, specific sequences may easily be replaced with another DNA fragment by the GATEWAY system. By placing the GATEWAY recombination sites at appropriate locations in the vectors, workload for subsequent cloning steps may be greatly reduced. Convenient binary vectors based on the GATEWAY technology have been reported by various authors (39,40).
3. Virulence genes. Small DNA fragments that contain *virE* or *virG* can improve the efficiency of transformation by a binary vector to some extent (4,5). A mutant *virG* gene, *virGN54D*, that is expressed constitutively in *Agrobacterium* cells gave much higher efficiency of transformation than wild types (41,42).

4. Device to suppress transfer of non-T-DNA segments.
 - a. Multiple left border repeats. Transfer of so-called “backbone sequences” from binary vectors to higher plants is not uncommon and has raised considerable concerns over genetically modified plants. A simple method is to place additional LB sequences close to the original LB; transfer of the backbone sequences is then suppressed in a nearly perfect fashion (43).
 - b. Killer gene. Another method is to place a gene, whose gene product is lethal to cells, outside the T-DNA to eliminate transformed cells that acquired the backbone (44).
5. Cosmid. The cloning capacity for a cosmid is up to about 50 kb, which includes the vector DNA, being based on the packaging system of phage lambda (14). A simple addition of one or two copies of cos sites can convert a binary vector to a cosmid.
6. P1 Cloning system. A P1 vector is more complex than a cosmid, and several components need to be integrated. However, P1 vector is very useful in genomic studies because the cloning capacity for a P1 vector is as large as 100 kb, being based on the packaging system of phage P1 (14).
7. Device for removal of selectable marker genes.
 - a. Cotransformation. Considerable concern has been raised over selectable marker genes in commercial transgenic plants. Cotransformation with two separate T-DNAs is a simple approach for removal of the marker gene. One T-DNA carries a selectable marker gene, and the other does genes of interest. There is a good chance that these T-DNAs, segregate independently, and marker-free progeny plants are identified. Two T-DNAs may easily be constructed on a super-binary vector (45). The only modification is that a T-DNA with a selectable marker gene is cloned into a precursor of an acceptor vector before the virulence fragment is inserted and an intermediate vector is prepared without a selectable marker gene.
 - b. Site-specific recombination systems. Many authors have reported vectors exploiting site-specific recombination systems derived from phages or fungi, such as the Cre-lox, Flp-FRT, and R/RS (46). In such a vector, a marker gene is flanked by the short target DNA sequences for a specific recombinase. After the integration of the T-DNA to plant cells, the recombinase is provided to the cells by various sophisticated means so that the marker gene is excised out.
8. Accommodation for very large DNA segments. For map-based cloning of plant genes, complementation tests of large genomic fragments provide key information. Single-copy vectors that carry Ri ori for *A. tumefaciens* and F ori or P1 ori for *E. coli* were specifically designed for transfer of very large DNA fragments to higher plants and designated as BIBAC (47) and TAC (48). On the other hand, because a simple IncP binary vector was able to maintain DNA fragments stably over 300 kb (49), conventional binary vectors that do not carry the ori of ColE1 or pUC may also be good for this purpose. We think the current situation is that cloning and transfer of DNA fragments larger than 50 kb is possible, but the efficiency is still low with any of these vectors.

3. Methods

3.1. Construction of a Typical Binary Vector

A standard flowchart showing the construction of binary vectors, from various components to the creation of an *empty vector*, a vector with a plant-selectable marker (*selection vector*), and finally a vector with both a reporter gene and a selectable marker (*reporter vector*) is illustrated in **Fig. 1**. The reporter gene in this flowchart may be a model for any genes of interest. In transformation experiments, a reporter vector may serve as a control vector, which gives reference points for virtually all important measurements in transformation processes, such as frequency of transformation, growth of transformed cells, efficiency of plant regeneration, growth of transgenic plants, phenotypes of plants, level of foreign gene expression, effects of genes of interest, and so on (see **Note 11**). Our recommendation is to start the consideration of experimental designs from the configuration of such a control vector. Quite often, a reporter vector may be a good starting material for various gene constructs, as experimental vectors may be obtained simply by replacing one or more components in the reporter vector with appropriate DNA fragments. Useful tips related to vector construction are given in **Notes 12–18**.

1. Obtain plasmids and other DNA fragments necessary for constructions of vectors from appropriate sources.
2. Combine the bacteria-selectable marker and the plasmid replication functions for *E. coli*.
3. Insert the plasmid replication functions for *A. tumefaciens*, if necessary.
4. Insert the plasmid mobilization functions, if necessary.
5. Insert the RB, the LB, and the MCS to give the empty vector.
6. Construct the expression unit of the selectable marker gene separately.
7. Insert the unit into the empty vector to give the selection vector.
8. Construct the expression unit of the reporter gene separately.
9. Insert the unit into the selection vector to give the reporter vector.

3.2. Examples of Binary Vectors

Some examples of binary vectors are shown in **Fig. 2**. Various derivatives differing in the selectable marker, reporter, MCS, and other factors are available in the pCambia series (www.cambia.org), pGreen series (**27**), and pPZP series (**50**). One of the derivatives is shown for each of the groups; empty vector pGreen0000, selection vector pPZP111, and reporter vector pCambia1302. A similar variation is found in the derivatives of pBin19, which is considered to be a selection vector, and reporter vector pBI121, shown in **Fig. 2**. An empty version of pBin19 may be obtained by digesting pBin19 with *Clal* and partially with *SacII*, followed by recircularization. These vector groups have been

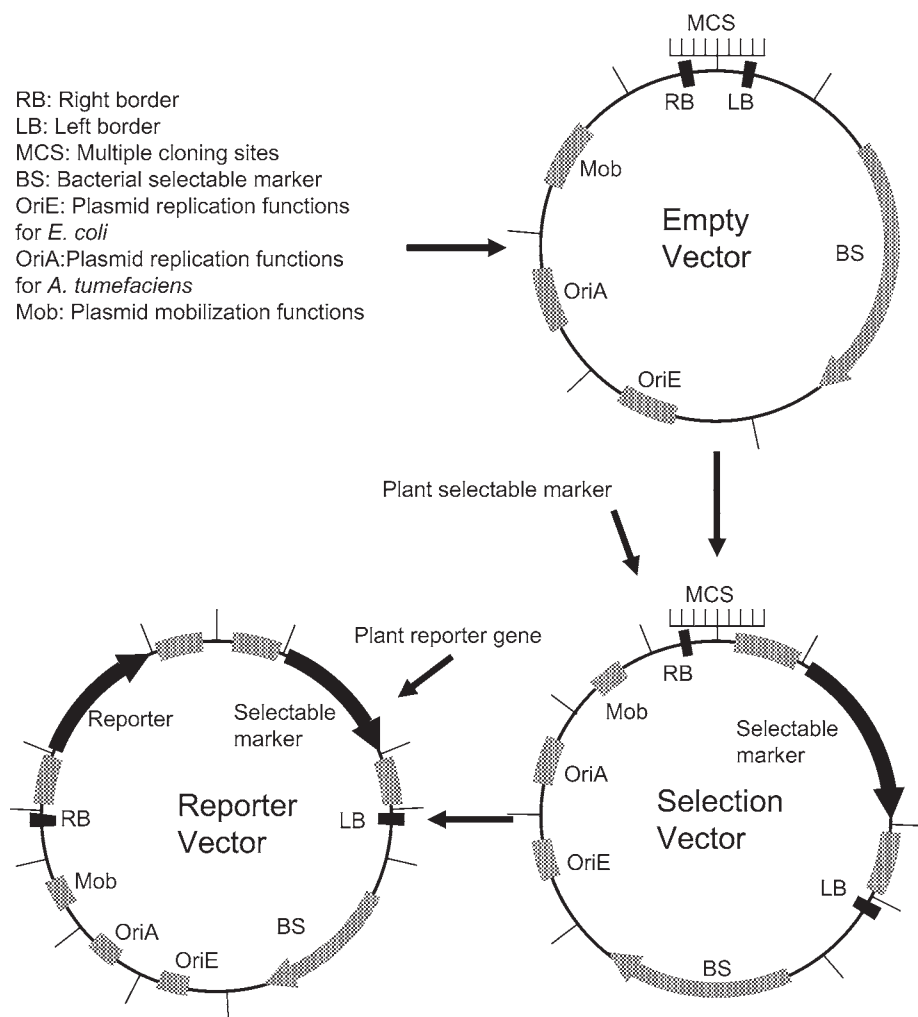


Fig. 1. A simplified flowchart showing the construction of binary vectors.

successfully employed in many studies, which, at a glance, account for two-thirds of the recent publications in the area of plant transformation.

3.3. Construction of a Typical Super-binary Vector and Examples

The system of a super-binary vector consists of two plasmids, an intermediate vector and an acceptor vector, and the final construct is a cointegrate plasmid created by homologous recombination in *A. tumefaciens* (45). Protocols for both a single T-DNA vector, in which a selectable marker and a gene of

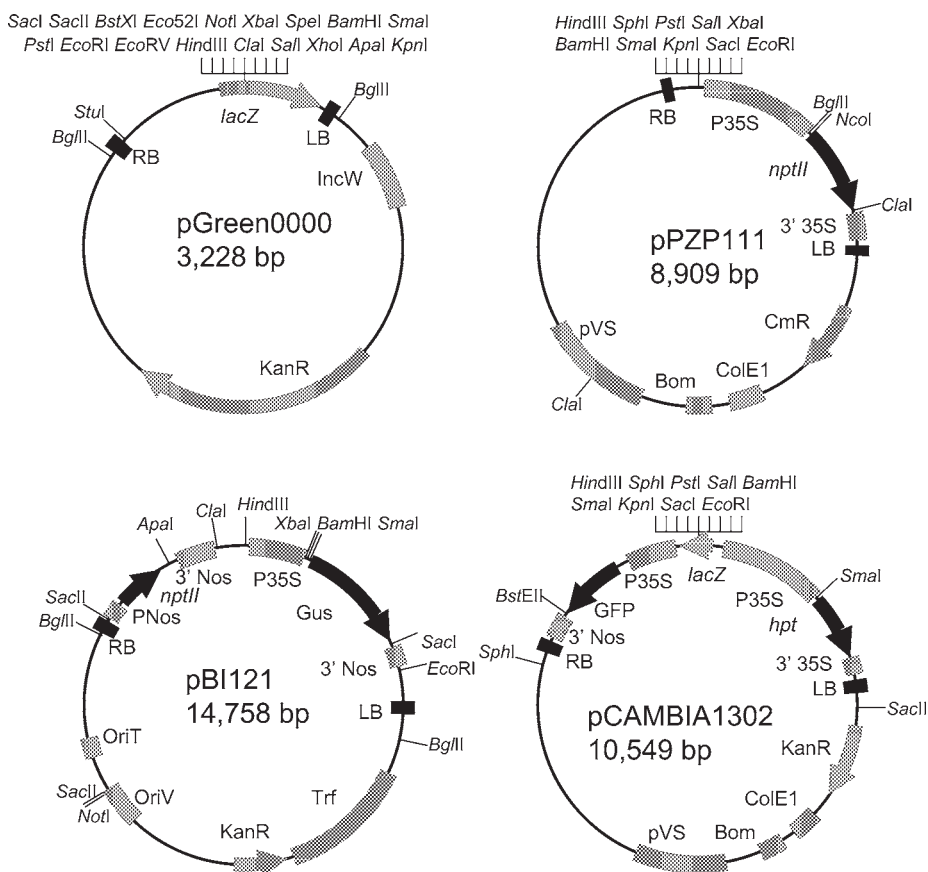


Fig. 2. Examples of binary vectors. The maps are based on sequences in GenBank, accessions numbers CVE7829 for pGreen0000, CVU10487 for pPZP111, AF485783 for pBI121, and AF234298 for pCambia1302. Abbreviations: RB, right border; LB, left border; P35S, promoter for 35S transcript; 3' 35S, 3' signal for 35S transcript; PNos, promoter for nopaline synthase; 3' Nos, 3' signal for nopaline synthase; *nptII*, neomycin phosphotransferase II; *Gus*, β -glucuronidase; *hpt*, hygromycin phosphotransferase; *lacZ*, α -subunit of β -galactosidase; *IncW*, origin of replication of *IncW* plasmid; *pVS*, origin of replication of *pVS1*; *OriV*, origin of vegetative replication of *IncP* plasmid; *Trf*, transacting replication function of *IncP* plasmid; *OriT*, origin of transfer of *IncP* plasmids; *ColE1*, origin of replication of *ColE1*; *Bom*, bom site for plasmid transfer of *ColE1*; *CmR*, chloramphenicol resistance gene; *KanR*, kanamycin resistance gene.

interest are linked in a T-DNA, and a double T-DNA vector for cotransformation are described here.

3.3.1. Single T-DNA Vector

1. Construct an empty intermediate vector by combining the Ori-Cos fragment, the spectinomycin resistance gene from Tn7, the cos site of phage lambda, the RB and the LB from pTiT37, and the MCS. pSB11 (**Fig. 3**) is an example.
2. Clone a gene of interest and a plant-selectable marker gene into the MCS of the empty vector.
3. As a preliminary step in the construction of an acceptor vector, combine the Ori-Cos fragment, the tetracycline resistance locus, OriV, Trf, OriT, and an MCS that consists of *Xba*I, *Sac*I, *Xho*I, *Kpn*I, and *Hind*III recognition sites to give a precursor plasmid. pNB1 is an example (**Fig. 3**).
4. Clone the Super-vir fragment into the *Kpn*I site of the precursor plasmid to give an acceptor vector (see **Note 19**). pSB1 (**Fig. 3**) is an example.
5. Introduce the derivative of the intermediate vector into an *A. tumefaciens* strain that carries the acceptor vector by triparental mating, and select a strain for spectinomycin and tetracycline resistance and growth on a minimal medium to create the cointegrate (see **Note 20**).

3.3.2. Double T-DNA Vector

1. Clone separately a gene of interest and a plant-selectable marker gene into an empty intermediate vector.
2. Cut out the T-DNA of the selectable marker from the intermediate vector as a *Sall* fragment and clone into the *Xho*I site of the precursor plasmid described in **Subheading 3.3.1**. (see **Note 21**).
3. Clone the Super-vir fragment into the derivative of the precursor plasmid to create an acceptor vector with the plant-selectable marker gene. pSB4U (**Fig. 2**) (**43**), which has the hygromycin resistance gene connected to the Ubi promoter and the Nos 3' signal, is an example.
4. Introduce the intermediate vector with the gene of interest into an *A. tumefaciens* strain that carries the acceptor vector with the plant-selectable marker gene by triparental mating and select a strain for spectinomycin and tetracycline resistance and growth on a minimal medium to create the cointegrate.

3.4. Preparation of *A. tumefaciens* with Binary and Super-binary Vectors Ready for Infection

After completion of molecular construction in *E. coli*, vectors are transferred to *A. tumefaciens* by the procedures described in Chapter 3 of this volume. For the construction of super-binary vectors, triparental mating is highly recommended (see **Note 20**).

Rearrangement of vectors may sometimes take place during the process of introduction of plasmids into *A. tumefaciens*. It is very important to confirm

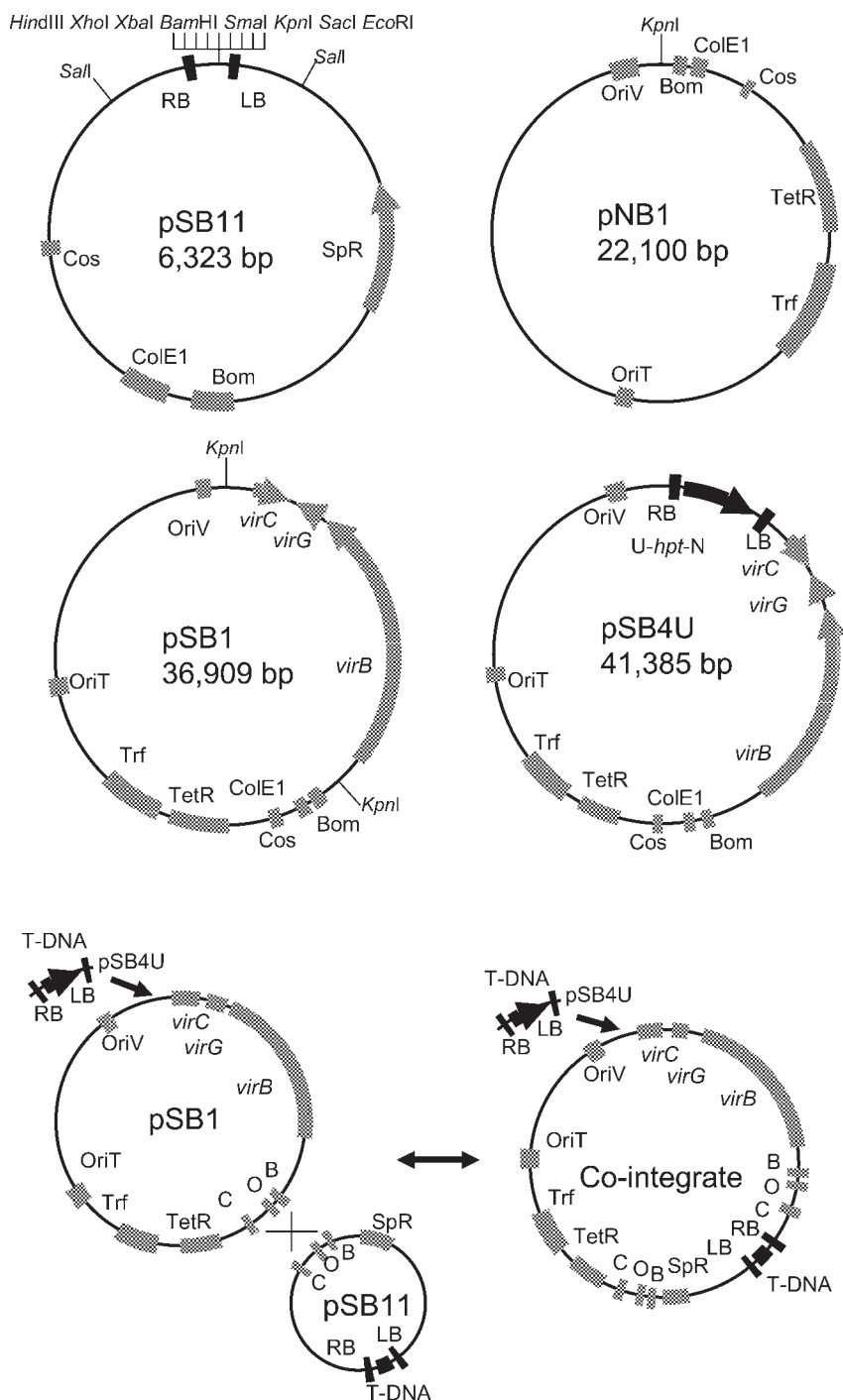


Fig. 3.

the structure of vectors in colonies of *A. tumefaciens* and select the colonies that carry the right vectors. Once the right colonies are identified, the vectors are reasonably stable in *A. tumefaciens*.

Ideally, plasmids are purified from *A. tumefaciens* and extensively characterized by restriction analysis. However, preparation of plasmids from *A. tumefaciens* is much less efficient than that from *E. coli*, and a certain amount of experience with this protocol is needed to obtain good preparations of the plasmids. Alternatively, amplification of a number of key fragments of the vectors by polymerase chain reaction (PCR) from the colonies of *A. tumefaciens* or Southern analysis of total DNA preparation from *A. tumefaciens* cells is performed.

If something is wrong with the transformation vector, years of time, effort, and precious resources can be wasted. A laboratory should establish a series of quality control (QC) protocols for vectors and bacterial strains (see **Note 22**). Ideally, QC protocols are written down, and a member of the laboratory is designated as the QC manager, who makes sure everyone follows the rules of the laboratory.

4. Notes

1. The capability of a super-binary vector is most evident when it is combined with strain LBA4404, whereas the performance of a super-binary vector is not very good when it is carried by strains derived from A281, such as EHA101, EHA105, or AGL1 (6,9). The *virC1* gene in the super-binary vector is probably inactive owing to a frame-shift mutation that took place during the construction of vectors (see GenBank accession number AB027255), but there is no evidence that this affects the efficiency of transformation.
2. Useful examples of the plasmids are listed below, but do not try to make a complete set at one time. It is good enough to create plasmids as required and let the library grow over time.
 - a. Minus-one vector: this vector lacks one of the components of the expression units of the vector.
 - b. Empty vector: the T-DNA of this vector has only MCS.

Fig. 3. (previous page) Examples of super-binary vectors and illustration of integration of an intermediate vector to an acceptor vector. The maps of pSB11, pSB1, and pSB4U are based on GenBank accession numbers AB027256, AB027255, and AB201314, respectively. Abbreviations: RB, right border; LB, left border; U-*hpt*-N, Ubiquitin promoter-hygromycin phosphotransferase-3' signal for nopaline synthase; OriV, origin of vegetative replication of IncP plasmid; ColE1 or O, origin of replication of ColE1; Trf, transacting replication function of IncP plasmid; OriT, origin of transfer of IncP plasmid; Bom or B, bom site for plasmid transfer of ColE1; Cos or C, Cos site of phage lambda; TetR, tetracycline resistance gene; SpR, spectinomycin resistance gene.

- c. High-copy MCS vector: a high-copy-number plasmid that has only MCS and no T-DNA borders. This may be convenient for creation of construction intermediates, especially in case the vector to be constructed is a low-copy-number plasmid
 - d. Marker cassette vector: the high-copy MCS vector that carries the expression unit of the plant-selectable marker gene.
 - e. Reporter cassette vector: the high-copy MCS vector that carries the expression unit of the reporter gene.
3. The T-DNA border regions of nopaline-type plasmids, pTiC58 and pTiT37, are almost identical, and the T-DNA sequences of the well-studied octopine-type Ti plasmids, pTi15955, pTiAch5, pTiA6, and pTiB6S3, are very similar to each other if not identical (51,52).
 4. In vectors constructed earlier, the selectable markers were located close to the RB. Because the transfer intermediate of the T-DNA is made in the direction from the RB to the LB, it is considered that deletion of a gene of interest may be prevented by placing a selectable marker close to the LB, and later most plasmids were constructed in this way. In contrast, integration of T-DNA into a plant chromosome is said to take place in the direction from the LB to the RB (53). We have observed deletions at both the right and left ends of the T-DNA in plants. Therefore, it is our opinion that the location of the selectable marker does not matter much.
 5. The use of weak promoters may not always be a bad idea. Strong promoters could waste resources for transcription and translation machinery in plant cells after transformation. In addition, because the levels of expression of marker genes and genes of interest are often linked, selection of transformants with weak selectable markers may cause strong expressers of the genes of interest to be obtained.
 6. Expression of the *nptII* gene can inactivate a group of aminoglycoside antibiotics (14). Choice of antibiotic is an important factor in plant transformation. For example, because kanamycin does not restrict growth of rice and maize cells, it is not used for transformation of these plants. Many transformed rice cells resistant to G418 were albinos. However, rice and maize can be transformed reasonably well with the *nptII* gene based on resistance to paromomycin (unpublished results).
 7. The DNA segments connected to the 3' ends of genes are often called terminators, but the terminology is sometimes confusing, because signals for the termination of transcription and for the addition of polyA sequences are different. Exact functions of many 3' sequences are not well characterized and it is not usually confirmed whether termination signals are really contained in terminators. Therefore, we use the term 3' signal in this chapter.
 8. Partly because the TATA boxes of eukaryotic promoters resemble prokaryotic promoters to some extent, many plant promoters are active in both *E. coli* and *A. tumefaciens* (35). The fact that both the widely used strain EHA101 and the vector pBin19 have kanamycin resistance had caused some inconvenience in earlier days. One solution was insertion of the 35S-*hpt* gene to pBin19, which gave

another resistance to the vector and made introduction of the vector to the strain easier.

9. Subcloning of a gene into IncP plasmids can sometimes be a less efficient practice compared with that of using the pUC derivatives. It is advisable to follow faithfully the cloning procedures and tips described in standard textbooks such as *Molecular Cloning* (14). Oversimplification or too many shortcuts during the subcloning process may lead to complications and delay the completion of construction. Sequence and other genetic information of IncP plasmids has been previously described (25). In general, IncP vectors that are larger than 20 kb can be more stably maintained in both *E. coli* and *A. tumefaciens* than smaller IncP vectors. We have observed that, after 3 d of culture without selective antibiotics, most of the *A. tumefaciens* cells retained large IncP plasmids, whereas more than half of the cells lost the small IncP vectors. How this phenomenon affects the transformation experiments is not known. This can be important for *Arabidopsis in planta* transformation, in which *A. tumefaciens* cells probably sit in plant tissues for some time before the gene transfer process takes place.
10. Although plasmid mobilization functions are not needed for transformation of *A. tumefaciens* by electroporation or freeze-thaw methods, it is a good idea to have broader options. When plasmid cointegration in *A. tumefaciens* is intended, triparental mating is much more efficient than electroporation, and these functions are necessary.
11. When plants are transformed with various gene constructs, it is a good idea to always transform plants in parallel with such a vector, which is extremely useful in monitoring many aspects of transformation processes. Many factors are involved in successful transformation, and it is not a simple task to maintain capability of plant transformation stably over time. If something goes wrong in experiments, what is happening in the control plots can answer many questions. It is a good idea to include the same control vector in all the transformation experiments conducted in a laboratory. We have been transforming rice for more than 15 years now with *A. tumefaciens*. We can still compare data between current and very early experiments if pIG121Hm (9) is included. Such a control vector could also play the role of a “spearhead”. New enabling technologies are continuously developed, and new methods for plant transformation are tested one after another. Each time, new factors are incorporated into mainstream protocols in a laboratory after they are tested extensively with the control vector.
12. Do not assume that external information related to biological materials is 100% correct. This is one of the hot spots for complications. You may receive incorrect or incomplete maps, sequence information, protocols, and even plasmids. The earlier you confirm materials and information after receipt, the more easily problems are identified and solved.
13. Characterize biological materials including plasmids and DNA fragments, and evaluate data collected in-house and external information as extensively as practical. Ideally, everything from external sources is fully sequenced in-house. At least, partial sequencing of the most critical segments and restriction analysis

with every six-base cutter enzyme available should be performed. The finished vectors should also be characterized as described here.

14. Simulate vector construction *in silico* and prepare sequence files and maps of the vectors to be constructed before starting wet laboratory practices.
15. All fragments amplified by PCR must be fully sequenced.
16. It should be noted that similar genes, which consist of the same promoters, the same coding sequences, and the same 3' signals, could still be expressed quite differently even in the same plant species when they are placed in different vectors, probably being affected by small differences in the configurations of the vectors. Because the nature of these effects is not well understood, it is a good idea to consider more than one molecular design and to use trial and error.
17. A prudent approach for experimental constructs is to make as few alterations as possible from a reporter vector, from which marker genes have been expressed very well.
18. In the design of vectors, avoidance of repeats of sequences is highly recommended.
19. The packaging extracts from phage lambda can greatly facilitate this cloning because the precursor is a cosmid and the size of the precursor plus the Super-vir fragment is good for the packaging reaction.
20. Triparental mating (see Chapter 3) is a simple technique in principle but is sometimes a hot spot of complications. The selection of *A. tumefaciens* from *E. coli* is often based on capability of growth on a minimal medium. A certain background growth of nontarget bacteria is inevitable on primary selection plates, but colonies that can grow as fast on the selective medium as on a nonselective medium are clearly distinguishable. Second selection plates are usually clean, but it is a good idea to perform one more selection culture. After the third selection, plasmids are prepared from the selected colonies as described in Chapter 3 in this volume, and restriction analysis is performed.
21. Although there are unique restriction sites in pSB1 and other acceptor vectors, direct cloning of additional DNA fragments into these vectors is not efficient. Therefore, if modification of an acceptor vector is necessary, it is a good idea to go back one step to a precursor plasmid like pNB1, which lacks the Super-vir fragment. After modification of the precursor plasmid, the 14.8 KpnI Super-vir fragment is cloned back. Again, the packaging extracts from phage lambda can facilitate this cloning. It should also be noted that pUC plasmids carrying the Super-vir fragment are sometimes unstable, and pSB1 is a good source of this fragment.
22. The following points should be addressed in standard QC protocols:
 - a. Source materials: it is a good idea to establish a central stock of a laboratory of commonly used plasmid DNA, bacteria, and other biological materials used for construction of vectors.
 - b. Constructed vectors and strains: DNA of finished vectors and all construction intermediates should be stored for a defined length of time. Purified plasmid DNA may be stored at -20°C for a very long time. Key plasmids are stored as DNA and *E. coli* with the plasmids.

- c. Finished vector: a standard process for quality checking of vectors ready for plant transformation should be established. Thorough restriction analysis with more than 10 enzymes and sequencing of junctions of fragments manipulated during the construction are minimum requirements. Important vectors should be fully sequenced.
- d. *A. tumefaciens* strain ready for transformation: the routine practice for confirmation of structures of vectors in *A. tumefaciens* and the method of storage of the strains should be described.
- e. Handling of *A. tumefaciens*: proper handling of bacterial strains recommended by bacteriologists may not always be exercised by current molecular biologists. For *E. coli*, which outgrows virtually any organisms in laboratories, this is usually not a problem, but for *A. tumefaciens*, practices like always using fresh, well-isolated colonies, whose shapes and colors are carefully examined, are important.
- f. PCR primers and probes: quite a few pairs of primers and probe fragments are used during the construction. These are also useful for confirmation of vector structure and analysis of transgenic plants. It is a good idea to organize a library of these materials in a laboratory.
- g. Bioinformatics: sequences, maps, and other data from various assays of the aforementioned materials constitute an enormous amount of electronic data, which should be well organized, regularly updated, and available to everybody in the laboratory.

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Three Methods for the Introduction of Foreign DNA into *Agrobacterium*

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Summary

The genetic manipulation of *Agrobacterium tumefaciens* is used to facilitate studies of bacterial gene functions or as a first step in introducing genetic material into transformable plant cells through the use of T-DNA binary vectors. Three methods are commonly used. Transformation with purified plasmid can be done with either electroporation or a simple freeze/thaw transformation method. Alternatively, a mobilizable plasmid can be placed into *Agrobacterium* using the triparental mating method. Here we present three detailed protocols for *Agrobacterium* strain construction using electroporation, the freeze/thaw method of transformation, and triparental mating.

Key Words: Electroporation; freeze/thaw; triparental mating; conjugation; incompatibility group; selective plates; counterselection; donor; recipient; transconjugant; self-transmissible plasmid; origin of transfer (*oriT*).

1. Introduction

The most efficient method of placing foreign DNA into *Agrobacterium* is electroporation. Electroporation depends on the use of electric shock to create aqueous pores in the lipid membrane of the bacteria. These pores are sufficiently large to allow DNA molecules (and other macromolecules) to enter the cell. At the same time, recovery of transformants is contingent on the capacity of the membrane to reassemble and allow cells to survive the shock. The success of the method depends on regulating the electric field strength and the duration of the pulse (1,2). These parameters are partly managed by adjusting settings on an electroporation apparatus to regulate the initial intensity of the electric field (kV/cm) and resistors (ohm setting) that determine the flow of current through the cells. The duration of the pulse (the time constant) is also strongly affected by the ionic strength of the cell/DNA mixture receiving the

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pulse. For that reason, “electro-competent” cells are prepared with thorough washing to remove electrolytes, and DNA must be in a salt-free solution. Electroporation is a highly efficient mechanism of transformation that can result in well over 10^6 transformants per μg circular plasmid DNA. We note that transformation efficiency declines with very large plasmids, but electroporation has been successfully used to introduce plasmids as large as 200 kb into *Agrobacterium* (3). The technique is sufficiently productive for use in constructing genomic libraries and may even be used when strain construction depends on the uptake and integration of linear DNA (4) or a nonreplicating plasmid.

When purified plasmid DNA is available, a fast and simple alternative to electroporation is the freeze/thaw transformation method (5,6). The exact mechanism by which this method works is not well understood. Presumably, the uptake of DNA relies on damaging the cell wall by exposure to divalent cations and rapid changes in temperature that alter the fluidity of the cell membrane. Estimations for efficiency for this method range from 10^2 to 10^3 transformants per μg DNA, several orders of magnitude lower than for electroporation. However, high transformation efficiency is not a primary consideration when one is simply moving a shuttle plasmid constructed in *E. coli* into *Agrobacterium*. The freeze/thaw method has the added benefit of not requiring specialized equipment.

Triparental mating is an effective method for moving a nonconjugative, but mobilizable plasmid into *Agrobacterium* (Fig. 1). The method makes use of two *E. coli* strains to move the plasmid of interest into *Agrobacterium*. The first *E. coli* strain carries a conjugative (self-transmissible) plasmid. This “helper” plasmid encodes all the proteins for formation of a mating bridge and transfer of itself or another mobilizable plasmid to recipient cells. The helper plasmid moves itself into the second *E. coli* strain (the donor strain), which carries (on a mobilizable plasmid) the genes one wishes to place into *Agrobacterium*. Trans-acting functions of the helper plasmid act to move the plasmid carried by the donor strain into *Agrobacterium* recipient cells. These trans-acting functions include single-strand nicking within the *oriT* (origin of transfer) region and targeting of the nicked DNA (the relaxosome) to the mating bridge that links donor and recipient cells (7).

The interaction between *oriT* of a mobilizable plasmid and the helper-supplied transfer functions is specific (7,8). If plasmids belong to the same incompatibility group, trans-acting helper enzymes will recognize *oriT* on the plasmid to be mobilized. Although two plasmids of the same incompatibility group will not be stably maintained in the same cell culture, the temporary presence of the helper plasmid is sufficient to transfer the mobilizable plasmid. Closely related

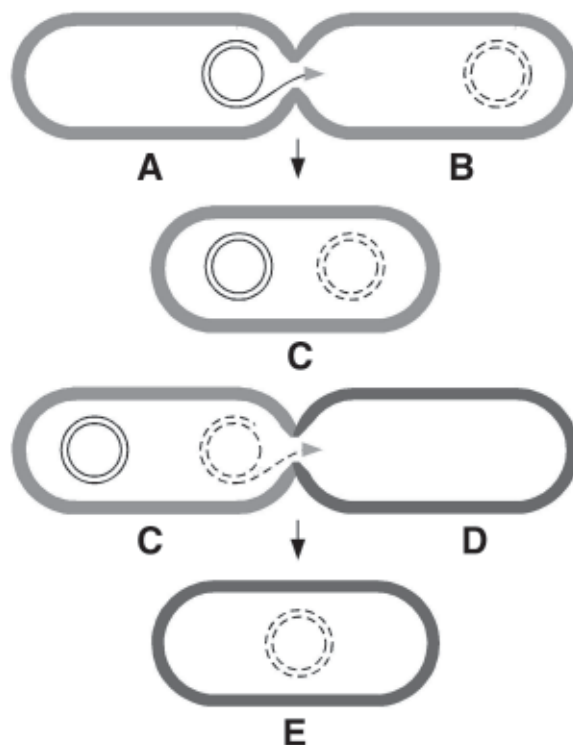


Fig. 1. Diagram depicting triparental mating. The *E. coli* helper and donor strains have been mixed together with the *Agrobacterium* recipient strain. **(A)** The *E. coli* helper strain transfers the self-transmissible plasmid pRK2013 (solid circles) to *E. coli* donor strain. **(B)** The *E. coli* donor strain carries an engineered plasmid that is mobilizable but not self-transmissible (dotted circles). **(C)** The donor strain acquires pRK2013 from the helper strain and now carries both plasmids. **(D)** Using transfer functions supplied by pRK2013, the donor strain transfers the engineered plasmid to the *Agrobacterium* recipient. **(E)** The engineered plasmid replicates and becomes established in the *Agrobacterium* cells.

IncP plasmids with cognate transfer and mobilization functions include derivatives of RK2, RP4, and R68. One widely used helper plasmid, pRK2013, carries the transfer and mobilization functions of the RK2 plasmid on a *ColE1* replicon (9,10). Thus, pRK2013 can move plasmids carrying an RK2-derived *oriT* from *E. coli* into *Agrobacterium* but cannot itself replicate in *Agrobacterium*. A selection of mobilizable T-DNA binary vectors that can be transferred to *Agrobacterium* through triparental mating with an *E. coli* strain containing pRK2013 is described by Hellens et al. (11).

2. Materials

2.1. Bacterial Strains (see Note 1)

1. *Agrobacterium* strain: EHA101 (12), EHA105 (13), LBA4404 (14), or other.
2. *E. coli* helper strain: HB101, DH5 α , or XL1-blue containing pRK2013 (available from the American Type Culture Collection).
3. *E. coli* donor strain: HB101, DH5 α , or XL1-blue containing engineered mobilizable plasmid.

2.2. Bacterial Media

1. MG/L medium: 5 g/L tryptone, 2.5 g/L yeast extract, 5 g/L NaCl, 5 g/L mannitol, 0.1 g/L MgSO₄·7H₂O, 0.25 g/L K₂HPO₄, 1.2 g/L L-glutamate, thiamine (10% solution, filter-sterilized).
 - a. Dissolve all ingredients except thiamine in 900 mL water.
 - b. Check that pH is close to 7.0.
 - c. Bring volume to 1 L.
 - d. For MG/L plates, add solution to 15 g/L agar.
 - e. Autoclave.
 - f. When media cools to 60°C or below, add 120 μ L/L of the thiamine solution and any antibiotics required for plasmid maintenance or selection.
2. YEB medium: 5 g/L tryptone, 1 g/L yeast extract, 5 g/L nutrient broth, 5 g/L sucrose, 0.49 g/L MgSO₄·7H₂O.
 - a. Dissolve ingredients in water.
 - b. Adjust the pH to 7.2 and bring the volume to 1 L.
 - c. For YEB plates, add solution to 15 g/L agar.
 - d. Autoclave.
 - e. When media cools to 60°C or below, add any antibiotics required for plasmid maintenance or selection.
3. LB medium: 10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract.
 - a. Dissolve ingredients in water.
 - b. Bring volume to 1 L.
 - c. Check that pH is close to 7.
 - d. For LB plate medium, add the solution to 15 g/L agar.
 - e. Autoclave.
 - f. When temperature has cooled to 60°C, add any antibiotics necessary for plasmid maintenance or selection and pour plates.
4. Minimal medium plates (500 mL) for selection of *Agrobacterium* transconjugants: prepare and autoclave separately: a 20X salt solution (20 g/L NH₄Cl, 6 g/L MgSO₄·7H₂O, 3 g/L KCl, 0.2 g/L CaCl₂, and 15 mg/L FeSO₄·7H₂O), a 500 mM phosphate solution (60 g/L K₂HPO₄ and 20 g/L NaH₂PO₄, pH 7.5), and a 25% glucose solution. Also prepare a buffered agar solution: dissolve 2.6 g Bis-Tris in 460 mL distilled-deionized water.

- a. Adjust pH to 7.0 with HCl.
- b. Add the buffer to 7.5 g agar and autoclave.
- c. Cool the autoclaved solutions to 60°C.
- d. To the buffered agar solution, add 25 mL of the 20X salt solution, 10 mL of phosphate solution, and 5 mL glucose.
- e. Mix with a gentle rolling action.
- f. Add antibiotics, if required for strain selection or maintenance.

2.3. Supplies and Solutions for *Agrobacterium* Transformation

2.3.1. For Electroporation Method

1. Sterile distilled-deionized water.
2. 10% Glycerol (autoclaved).
3. Electropulse generator (Bio-Rad, Gene Pulser II).
4. 0.2-cm Electroporation cuvette (Bio-Rad, cat. no. 1652086).
5. Sterile Pasteur pipet and rubber bulb.

2.3.2. For Freeze/Thaw Method

1. 20 mM CaCl₂ (autoclaved).
2. Liquid nitrogen.

2.3.3. For Triparental Mating Method

1. 0.8% NaCl (autoclaved).
2. Cellulose membrane disk (pore size 0.45 µm, Whatman, cat. no. 140618).

3. Methods

3.1. Transformation of *Agrobacterium* through Electroporation

3.1.1. Preparation of Electro-Competent *Agrobacterium* Cells

1. Inoculate two glass culture tubes (16 × 125 mm) each containing 2 mL MG/L liquid medium, YEB, or other nutrient-rich *Agrobacterium* growth media, with single colonies of the *Agrobacterium* strain to be transformed. Include any antibiotic required for maintenance of an already present plasmid in the growth medium. Grow the cells overnight at 25–28°C with shaking to aerate.
2. Use the overnight cultures to inoculate two 1-L flasks each containing 200 mL of MG/L, YEB, or other nutrient-rich *Agrobacterium* growth medium (*see Note 2*). Include any necessary antibiotic. Incubate cells at 25–28°C with shaking for good aeration (220–250 rpm) until the OD₆₀₀ of the culture is between 0.5 and 1.5 (*see Note 3*.)
3. Chill cells on ice and transfer to four 250-mL sterilized centrifuge bottles. Centrifuge at 10,000*g* for 10 min at 4°C in a GSA or similar rotor. Pour off supernatant.
4. Wash cells with 20 mL per bottle of ice-cold sterile water. Vortex well to resuspend cells. Centrifuge at 10,000*g* for 10 min at 4°C. Remove supernatant. Repeat this washing step one more time.

5. Repeat the wash step using ice-cold 10% glycerol instead of water. Cells can now be resuspended in 400 to 800 μL cold 10% glycerol (see **Notes 4** and **5**).
6. Place 40- μL aliquots of the competent cells into individual tubes and store at -80°C . This method will produce sufficient electro-competent cells for 30 or more individual transformations.

3.1.2. Transformation of *Agrobacterium* through Electroporation

1. Prepare for the electroporation process by first adjusting the electroporation apparatus settings to 2.5 kV, 25 μF capacitance, and 400 Ω resistance, or follow the instructions of your electroporator if it has an *Agrobacterium* transformation program.
2. Have a glass culture tube (16 $\times$ 125 mm) containing 2 mL nutrient-rich growth medium such as MG/L or YEB (no antibiotics) available for growth and recovery of postpulse *Agrobacterium* cells. In addition have a small amount (about 300 μL) of the medium ready in a microcentrifuge tube.
3. Place a tube (40 μL) of electro-competent cells, your DNA sample (see **Note 6**), and a 0.2-cm gap electroporation cuvet (see **Note 7**) on ice.
4. Add 1 to 5 μL DNA (see **Note 6**) to the cells and transfer cell/DNA mix to the chilled cuvet (see **Note 8**). Dry the cuvet with a Kimwipe and fit it snugly into the cuvet holder.
5. Simultaneously press the two pulse buttons to deliver the electric pulse. Press the “time constant” button to display duration of the pulse (see **Note 9**). If you have an electroporator with an *Agrobacterium* transformation program, simply follow the manufacturer’s instruction.
6. Immediately add 300 μL of the growth medium to the cuvet (see **Note 10**). Using a Pasteur pipet, transfer the cuvet contents to the glass culture tube (16 $\times$ 125 mm) containing 2 mL *Agrobacterium* growth medium. Incubate the tube at 25–28 $^{\circ}\text{C}$ for 2 to 4 h with shaking.
7. Select for transformants by plating cells on media that contain the appropriate antibiotic(s) to select for your plasmid (see **Note 11**).

3.2. Transformation of *Agrobacterium* Using the Freeze/Thaw Method

3.2.1. Preparation of Freeze/Thaw-Competent *Agrobacterium* Cells

1. Inoculate 2 mL of liquid growth medium (e.g., MG/L or YEB) with the *Agrobacterium* strain that is to be transformed. Include any antibiotic required for maintenance of an already present plasmid. Grow the cells overnight with shaking to aerate at 25 to 28 $^{\circ}\text{C}$.
2. Use the 2-mL culture to inoculate 50 mL of the same medium in a 250-mL flask. Continue incubation until cells reach an OD_{600} between 0.5 and 1.0. Chill the culture on ice.
3. Pellet the cells by centrifuging at 4 $^{\circ}\text{C}$ for 8 to 10 min at 10,000 g in an SS34 or similar rotor. Discard the supernatant. Resuspend cells in 5 mL cold 20 mM CaCl_2 and repeat centrifugation. Discard the supernatant (see **Note 12**).

4. Resuspend the cells in 1 mL cold 20 mM CaCl₂. Aliquot 100–150 μ L of the cell solution into the chilled Eppendorf tubes. Store extra tubes of cells at –80°C for future use.

3.2.2. Transformation of *Agrobacterium* through Freeze/Thaw Method

1. Have purified plasmid DNA at a concentration of 0.1 to 1 μ g per μ L ready. Prepare a culture tube with 2 mL liquid growth medium (no antibiotics).
2. Retrieve frozen competent cells from freezer and place them on ice. Add 1 μ g plasmid DNA to cells before thawing (see **Note 13**).
3. Freeze the cell/DNA mix by lowering the tube into liquid nitrogen for about 5 min (see **Note 14**). Use a long-handled tweezers to hold the tube during the freezing process.
4. Thaw the frozen cell/DNA mixture for 5 to 10 min at room temperature. Transfer cells to the culture tube containing the 2 mL of growth medium and incubate with shaking at 25–28°C for 2 to 4 h.
5. Pellet the cells by spinning for 2 min at high speed in a microcentrifuge. Resuspend cells in 0.1–1 mL liquid growth medium containing the antibiotic to be used in selection of the plasmid (see **Note 15**). Plate 100 to 300 μ L cells on an agar plate containing antibiotics appropriate for selection of transformants.

3.3. Triparental Mating

1. Grow the *Agrobacterium* “recipient” strain (see **Note 1**) in 2 mL of a nutrient-rich growth medium (e.g., MG/L or YEB) with shaking to aerate at 25–28°C overnight.
2. Grow the *E. coli* “helper” strain (see **Note 1**) containing plasmid pRK2013 in 2 mL LB with kanamycin (50 μ g/mL) and the “donor” *E. coli* strain containing your mobilizable plasmid construct in 2 mL LB with appropriate antibiotic(s) overnight at 37°C, with shaking to aerate.
3. Dilute each *E. coli* culture 1:100 in LB with the appropriate antibiotics. Dilute the *Agrobacterium* strain 1:10 in growth medium. Continue to incubate the bacteria for an additional 4 h or so until the OD₆₀₀ for all strains is between 0.4 and 0.6.
4. Centrifuge 250 μ L of each culture in separate 1.5-mL microcentrifuge tubes for 2 min at 10,000*g*. Pour off supernatants and resuspend each cell pellet in 0.5 mL 0.8% NaCl. Pellet the cells again and decant the wash supernatant. Resuspend each cell pellet in 100 μ L of 0.8% NaCl.
5. Mix 100 μ L each of the donor and helper strains with 100 μ L of the *Agrobacterium* recipient strain in a microcentrifuge tube.
6. Place a sterile 0.45- μ m nitrocellulose disk on an LB plate (or other agar plate that allows good growth of both *E. coli* and *Agrobacterium*) without antibiotics. Place 100 μ L of the mixed cells on top of the filter (see **Note 16**). Incubate the plate for 2 d at 25–28°C.
7. After incubation, use a sterile tweezers to remove the disk from the plate into a 1.5-mL microcentrifuge tube. Add 0.5 to 1.0 mL 0.8% NaCl and vortex to resuspend cells (see **Note 17**).

8. Spread 100–200 μL of the cell suspension on an agar plate that contains antibiotics that will select for growth of *Agrobacterium* transconjugants carrying your plasmid construct and prevent growth of the helper and donor strains (see **Note 18**).
9. Incubate the selective plates for 2–4 d at 25–28°C until colonies form (see **Note 19**).

4. Notes

1. The choice of *Agrobacterium* recipient strain will depend on the goal of your experiment and the characteristics of the plasmid to be transferred. Hellens et al. (11) provide a useful overview (chromosomal background, antibiotic resistances, type of Ti plasmid, if any) for disarmed (avirulent) *Agrobacterium* strains that can be used as recipient cells when the eventual goal of the experiment is *Agrobacterium*-mediated plant transformation. A number of common *E. coli* lab strains can serve as helper and donor strains in the triparental mating procedure. The *E. coli* strains should be *recA* to prevent recombination between helper and donor plasmids that carry similar regions of DNA sequence. The use of strains carrying *hsdR* or *mcr* mutations improves the efficiency of triparental mating by disabling the natural restriction systems that *E. coli* uses to target foreign DNA. In addition, during the final steps of the triparental mating procedure, one can select against auxotrophic *E. coli* strains such as HB101, DH5 α , and XL1-blue by plating the conjugal cell mixture on minimal medium, provided your *Agrobacterium* strain will grow on that medium.
2. Electro-competent cells can be prepared in quantities sufficient for numerous transformation procedures and frozen indefinitely at –80°C.
3. If cultures are inoculated early in the day, they can be processed or partially processed on the same day. It may take 8 or more hours to reach an OD₆₀₀ of 1.0. There is some controversy as to the effect of growth phase on the electro-competence of *Agrobacterium*. Some researchers find that growth phase is an important factor (15) and harvest cells in the middle of the exponential growth phase. Others use cells that have reached early stationary phase with good results (2).
4. The purpose of the washing process is to remove all salts thoroughly, thus creating a solution of low ionic strength. Cell survival rate is enhanced by keeping cells and solutions well chilled during the washing process. Also, note that 10% glycerol may be used for all washes. Some believe that transformation efficiency is enhanced by letting the cells set at 4°C overnight following the first or second wash step. In any case, overnight refrigeration in the wash medium does not seriously impair transformation efficiency.
5. For optimal transformation efficiency, the final solution of electro-competent cells should be about 500-fold more concentrated than the starting culture volume. However, it can be difficult to remove the wash supernatants completely from the soft pellets without sacrificing cells. Thus, the glycerol concentration is maintained by using 10% glycerol for the final wash. In addition, the resuspension volume can be adjusted depending on how “wet” the pellet seems.
6. The DNA used in the transformation must be in a solution of low ionic strength. Plasmid DNA can be prepared with a commercially available kit or one of sev-

eral miniprep alkaline-lysis methods. One way to ensure the low ionic strength of the DNA solution is to precipitate a quantity of DNA with glycogen, a small quantity of salt, and ethanol. Example:

- a. Add 1 μL glycogen (20 mg/mL) and 1 μL 3 M NaOAc, pH 5.5 to 50 μL of miniprep or kit-prepared DNA.
 - b. Vortex lightly.
 - c. Add 150 μL absolute ethanol, mix well, and freeze at -20°C for 2 or 3 h.
 - d. Precipitate the DNA by centrifuging at 12,000g at 4°C for 20 min.
 - e. Wash two times with 1 mL 80% ethanol.
 - f. When the DNA is dry, resuspend it in sterile deionized water.
 - g. Plasmid DNA may be further diluted to between 0.05 and 10 ng per μL .
 - h. For transformation with genomic DNA, the concentration of DNA should be between 0.2 and 2 μg per μL (4).
7. The gap size describes the distance between the metal plates within the cuvette that act as electrodes during delivery of the electric shock. The electro-competent cells can be thawed at room temperature—for 2–3 min—and then immediately chilled on ice.
 8. Transfer to the cuvet will result in sufficient mixing of DNA and cells. Be sure that cells contact both of the metal plates within the cuvet.
 9. The time constant should be approximately 7–8 ms but may be a bit higher or lower, depending on your individual machine and the conductivity of the cell/DNA solution. If the electric pulse moves through the cuvet too fast, arcing of the electric current will result in a loud popping sound. This is usually an indication that the cell or DNA solution contains salt. Arcing dramatically increases cell death and severely lessens the likelihood of obtaining viable transformants.
 10. Cell recovery, and thus transformation efficiency, are improved by the immediate addition of growth medium to cells following the electric shock (1).
 11. The amount of cell solution to plate will vary depending on the experiment. If the intent of the transformation is merely to move a plasmid from *E. coli* into *Agrobacterium*, one can generally get good results from plating 100 μL of a 10^{-2} to 10^{-4} dilution of the cells. When selecting for a strain resulting from the integration of genomic or other nonreplicating DNA, it may be necessary to concentrate the cells and try plating amounts ranging from 5 to 100% of the cells.
 12. Some protocols for freeze/thaw transformation skip the wash step. However, if you have added antibiotics to your growth medium, this step will remove them and may aid recovery of transformants.
 13. One μg DNA is commonly recommended for freeze/thaw transformation, but some researchers use as much as 10 μg DNA per tube of cells.
 14. Dry ice and ethanol can also be used to freeze the cells. However, the use of liquid nitrogen may increase transformation efficiency (6).
 15. In general, between 10 and 100% of the transformed cells will be plated on a single selective plate. Addition of the selective antibiotic to the resuspension

medium helps prevent the formation of a layer of nontransformed cells that sometimes occurs when a thick resuspension of cells is plated on selective medium.

16. The cells will remain on the filter for ease of handling in the subsequent step and the liquid will be absorbed into the plate. However, if a nitrocellulose disk is not available, one can spot the cell mixture culture directly onto the agar plate.
17. If the mating mixture was plated directly onto the plate surface, scrape up the cells and resuspend them in 0.5 mL 0.8% NaCl.
18. A number of “disarmed” *Agrobacterium* strains intended for use with binary Ti vectors in plant transformation are resistant to rifampicin (usually 10 µg/mL) and/or other antibiotics (11). Media plates used in selection of the *Agrobacterium* transconjugants should be prepared with the antibiotics that select for your *Agrobacterium* strain as well as the antibiotic that will select for the presence of your engineered plasmid. If your *Agrobacterium* recipient does not carry an antibiotic selection marker, plate the conjugal cell mix on a minimal medium plate that contains the antibiotic for selection of the plasmid transferred to *Agrobacterium* from the donor *E. coli* strain. A prototrophic *Agrobacterium* strain will grow on the minimal medium. However, if the *E. coli* helper and donor strains are auxotrophs with special nutritional needs (see **Note 1**), minimal medium will suffice as a counterselection against them.
19. To confirm that colonies are not *E. coli* or *Agrobacterium* contaminated by *E. coli*, pick a single colony to streak duplicate selective plates. Incubate one plate at 37°C overnight and one plate at 25 to 28°C. If the colony was contaminated by *E. coli*, colonies will form overnight at 37°C, whereas *Agrobacterium* will not. Continue restreaking single colonies from the plate incubated at 25°C onto duplicate plates until there is no further sign of *E. coli* contamination.

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Integration of Genes into the Chromosome of *Agrobacterium tumefaciens* C58

Lan-Ying Lee

Summary

Agrobacterium tumefaciens has been widely used to transform numerous plant species. Frequently, investigators want to place more than one gene into *Agrobacterium* in order to manipulate various bacterial functions during plant genetic transformation. These genes are frequently brought into the bacterium by multiple plasmids. It is difficult to maintain several plasmids in the same bacterium without the use of a variety of antibiotics. However, the use of some antibiotics is not feasible for certain *Agrobacterium* strains. Also, too many different antibiotics in the culture medium generally results in slow growth of *Agrobacterium*. Therefore, it is to one's advantage to place genes of interest onto the bacterial chromosome to eliminate the use of multiple antibiotics during bacterial growth. This chapter describes in detail a method to integrate a gene of interest into the *pgl/picA* locus of the *Agrobacterium tumefaciens* C58 chromosome. The integrated gene is stably maintained as single copy per cell without the need for selection.

Key Words: *Agrobacterium tumefaciens*; plant transformation; gene integration; *pgl/picA* locus; single copy.

1. Introduction

During genetic manipulation, researchers often introduce multiple different genes into a single bacterium. Normally, these genes are carried by different plasmids. Because of the nature of incompatibility (Inc), plasmids within the same Inc group (i.e., with the same replication origin) exclude each other (cannot coreside) from the same bacterial cell. Considering the limited number of plasmids from different Inc groups available to replicate in *Agrobacterium*, the introduction of multiple genes of interest (GOI) may become difficult. To maintain resident plasmids stably, selection pressure, such as adding antibiotics in

the culture medium, is frequently applied. However, the use of too many different antibiotics can retard the growth of the bacteria. Such growth retardation may affect some of the physiological functions of the bacterium. In addition, sometimes one may want to keep the level of expression of the GOI low in order to minimize any effects of the new gene on bacterial growth or virulence. One preferred strategy is to use a low-copy-number replicon to carry the GOI or to use a promoter of low strength to express the GOI. Considering the points mentioned above, the integration of one's GOI into the bacterial chromosome may be most effective.

In the past, scientists frequently introduced a genetic element into the *Agrobacterium* chromosome to disrupt particular target genes (1,2). Hoekema et al. (3) and Miranda et al. (4) placed the T-DNA into the *Agrobacterium* chromosome and demonstrated that T-DNA could still be delivered into plants. The chromosomal insertion site of the genetic element must not affect either bacterial virulence or growth rate. Rong et al. (5,6) identified and characterized a plant-inducible locus on the *Agrobacterium* chromosome (*picA*) that is not required either for bacterial growth or for T-DNA transfer. The *picA* and neighboring *pgl* (polygalacturonase-like) genes are located on a 3.15-kbp *EcoRI* fragment of the chromosome of *A. tumefaciens* C58. A *PstI* site exists between these two genes. Insertion of a gene into this *PstI* site leaves enough length of DNA sequence on both sides (1.3 and 1.8 kb, respectively) to allow efficient double homologous recombination into the bacterial chromosome. This *EcoRI* fragment was cloned onto an IncP α plasmid, pLAFR1 (7), to make a basic integration vector, pE578 (Fig. 1). Previously, we inserted various genes into this *PstI* site and introduced them into the chromosome of strain C58 (8). To facilitate integration of the GOI into the *Agrobacterium* chromosome, we modified the basic vector and added several useful features. We thus constructed several versatile vectors (Fig. 1) that can be used to introduce any gene into the *pgl/picA* locus of the *Agrobacterium* C58 chromosome (9).

Figure 1 shows the various groups of vectors available in our laboratory. Plasmids pE1770 and pE1773 contain a *lacZ* α -complementation fragment that has eight unique restriction enzyme sites (*KpnI*, *HindIII*, *EcoRI*, *PstI*, *BamHI*, *SpeI*, *XbaI*, and *SacI*) between the *picA* and *pgl* genes. Using blue-white colony screening, one can use these sites to clone a gene and a selectable antibiotic resistance marker into these sites. The antibiotic resistance marker is used as an indicator of gene replacement in subsequent steps of the procedure. Alternatively, one can clone the GOI into a cloning vector containing a *ColEI* replication *ori* (such as pUC or pBluescript) first, and then cointegrate the entire plasmid into one of the unique restriction enzyme sites in the *lacZ* α -complementation fragment. The β -lactamase (ampicillin/carbenicillin resistance) gene on the backbone of this cloning plasmid can also be used as a selection marker

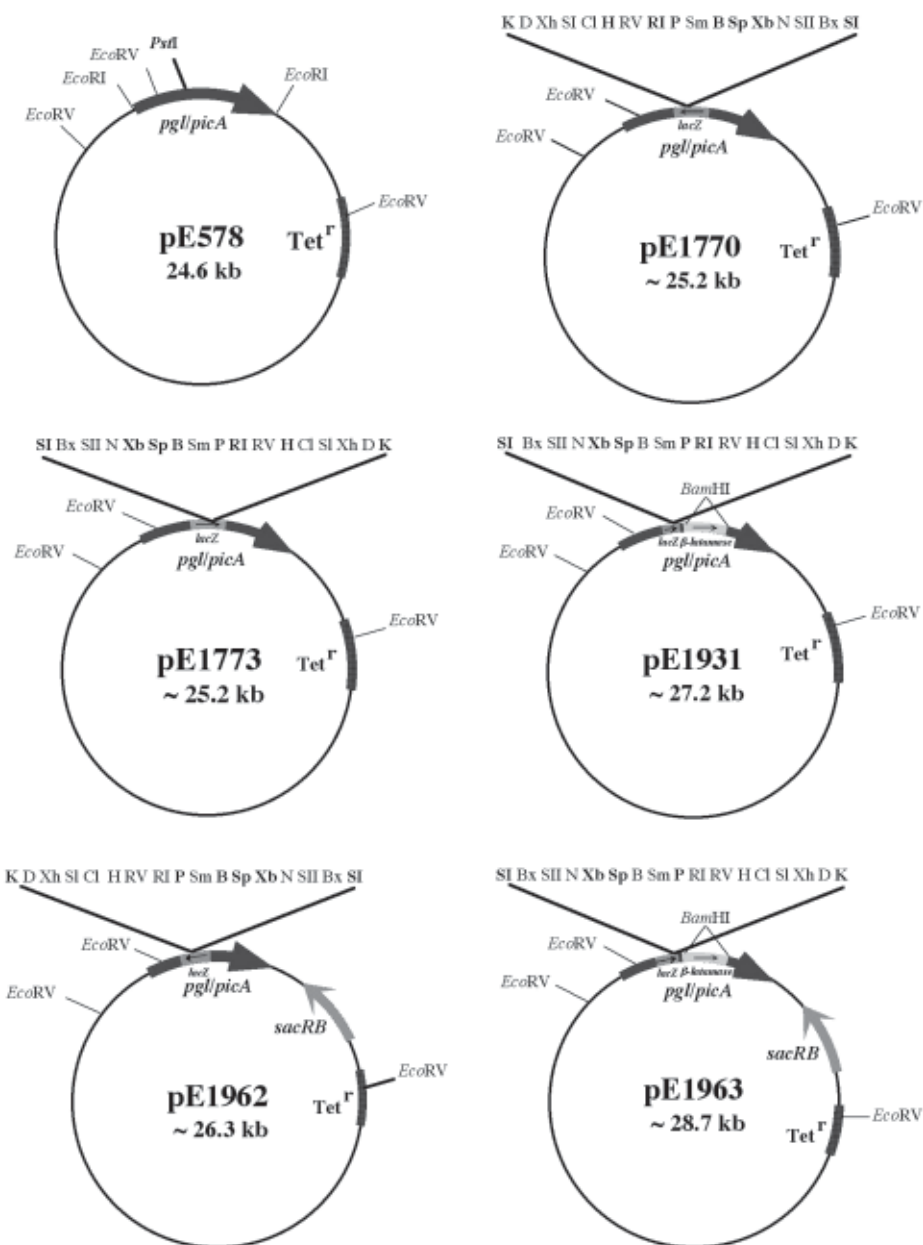


Fig. 1. Maps of the *Agrobacterium* integration vectors described in the text. The plasmids are not necessarily drawn to scale. Restriction enzyme sites in bold are unique on the plasmids. B, *Bam*HI; Bg, *Bgl*II; Bx, *Bst*XI; Cl, *Cl*aI; D, *Dra*I; H, *Hind*III; K, *Kpn*I; N, *Not*I; P, *Pst*I; RI, *Eco*RI; RV, *Eco*RV; SI, *Sac*I; SII, *Sac*II; SIII, *Sac*III; Sm, *Sma*I; Sp, *Spe*I; Xb, *Xba*I; Xh, *Xho*I; Tet^r, tetracycline resistance gene; *lacZ*, β-galactosidase α-complementation fragment; *pgl/picA*, polygalacturonase-like gene/plant-inducible locus on *Agrobacterium* chromosome; *sacRB*, *sacR* (regulatory gene), and *sacB* (levansucrase gene) of *Bacillus subtilis*.

to determine whether the GOI has integrated onto the *Agrobacterium* chromosome. In another series of vectors, we inserted a β -lactamase gene into the *pgl/picA* locus to allow for the introduction of only one's GOI. Such plasmids are pE1931 and pE1963 (**Fig. 1**). The third group of plasmids, pE1962 and pE1963, include the *sacR* and *sacB* genes from *Bacillus subtilis* (**10**) on the plasmid backbones. Because the *sacB* gene product metabolizes sucrose to a toxic compound, one can therefore use sucrose-containing medium to select for double crossover recombinants (and the loss of the *sacB*-containing vector) at the *pgl/picA* locus of *A. tumefaciens* C58. Blue-white colony screening can also be used to clone one's GOI into pE1963 or to clone both a GOI and a selection marker into pE1962.

To perform the chromosomal integration (**Fig. 2**) using the strategy first developed by Ruvkin and Ausubel (**11**), one places the GOI between the *pgl* and *picA* genes on one of the vectors mentioned above to make the "Starting Construction". Next, one introduces the Starting Construction into *A. tumefaciens* C58 either by bacterial conjugation (triparental mating), transformation, or electroporation to make the Starting Strain. An eviction plasmid belonging to the IncP α group, such as pPH1JI (**12**) or pVK102 (**13**), is then introduced into the Starting Strain by bacterial mating. Transconjugants that are resistant to both, the antibiotic marker introduced near the GOI and the antibiotic resistance marker on the backbone of the eviction plasmid (gentamicin resistance for pPH1JI or kanamycin resistance for pVK102), are selected. Because the integration vectors and the eviction plasmids belong to the same IncP α incompatibility group, they cannot coreside as replicons in the same bacterial cell. These transconjugants, which are resistant to both antibiotics, will fall into two possible categories. The first category includes bacteria that contain the entire starting plasmid integrated into the chromosome DNA through a single crossover recombination event (a plasmid cointegrate). The second category includes bacteria with the GOI and selection marker exchanged into the *pgl/picA* locus through double homologous recombination.

One then needs to check whether these transconjugants are resistant to the antibiotic encoded by the resistance marker on the backbone of the Starting Construction (for these plasmids, tetracycline). If the transconjugant is resistant to tetracycline, this indicates that the strain has the entire Starting Construction integrated into the chromosome. One should discard this type of transconjugant. Only those transconjugants that are sensitive to tetracycline are kept. Finally, one needs to extract total genomic DNA from these transconjugants. Following digestion with the appropriate restriction enzymes and Southern blot analysis, you can determine whether your GOI and antibiotic resistance markers have been exchanged into the *pgl/picA* locus on the chromosome. (**Fig. 3**). The GOI is stably maintained in this *Agrobacterium* strain at a single copy per cell without applying selection pressure.

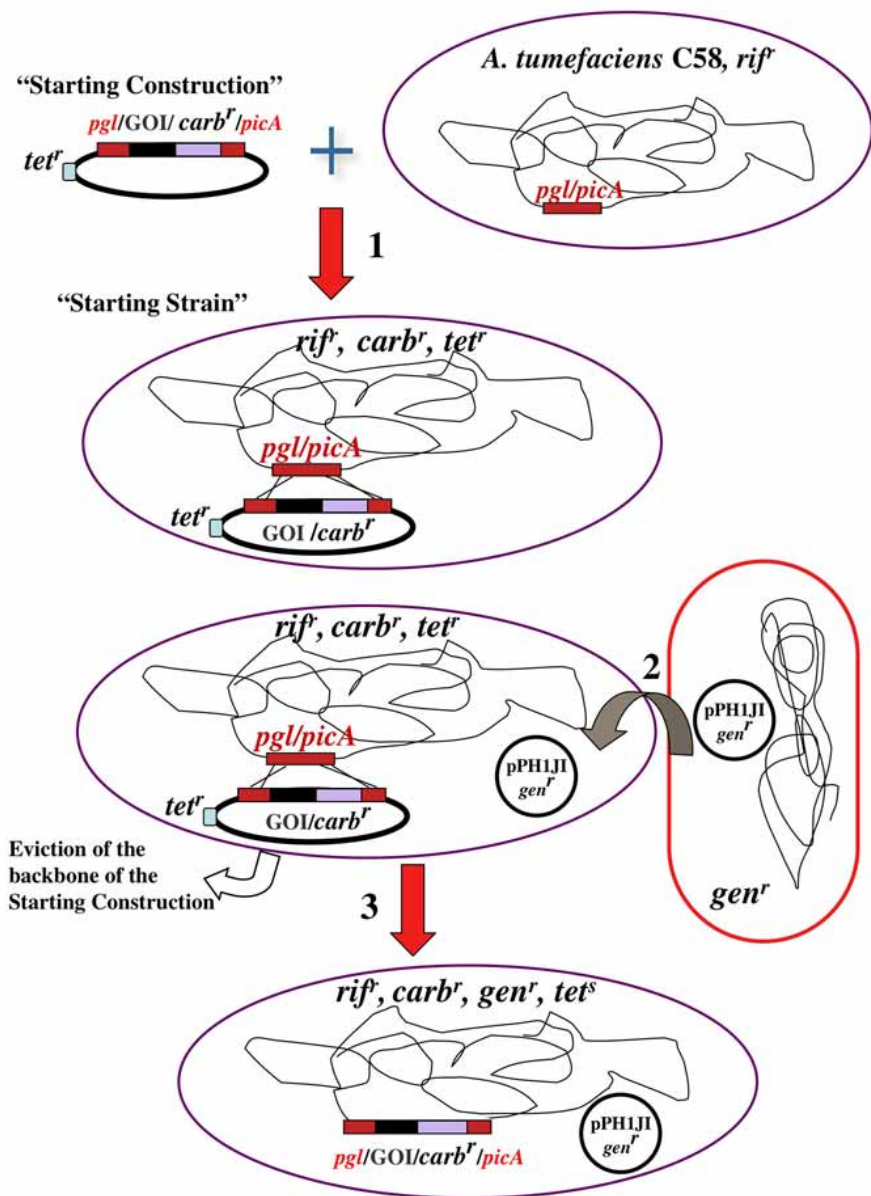


Fig. 2. Schematic representation of the experimental design for placing a gene of interest into the *pgl/picA* region of the *A. tumefaciens* C58. **Step 1:** Introduction of the Starting Construction into *A. tumefaciens* C58 using bacterial mating or transformation. **Step 2:** Introduction of an eviction plasmid by bacterial conjugation. **Step 3:** Selection of the final transconjugants. GOI, gene of interest; *carb^r*, carbenicillin resistance gene; *gen^r*, gentamicin resistance gene; *tet^r*, tetracycline resistance gene; *rif^r*, rifampicin resistance gene; *pgl*, polygalacturonase-like gene; *picA*, plant-inducible locus on *Agrobacterium* chromosome.

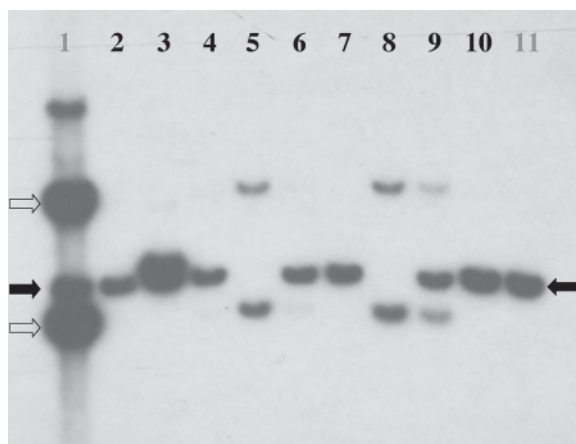


Fig. 3. Example of Southern hybridization to verify the integration of the GOI into the chromosomal *pgl/picA* locus of *A. tumefaciens*. Total genomic DNA extracted from various *A. tumefaciens* strains was digested with *EcoRI* and blotted onto a nylon membrane. A labeled 3.15-kbp DNA probe containing the *pgl/picA* locus was used to confirm the disruption of the *pgl/picA* locus in various *A. tumefaciens* strains. Lane 1, *A. tumefaciens* strain C58 containing the Starting Construction. Lanes 2–10 are nine individual clones that may or may not have the integration of the GOI. Lane 11, *A. tumefaciens* strain C58. Solid arrows indicate the intact 3.15-kbp *pgl/picA* locus from the wild-type *A. tumefaciens* chromosome (lanes 1 and 11). Open arrows indicate DNA fragments from the disrupted *pgl/picA* locus on the Starting Construction (lane 1).

Note that the intensity of the DNA band from the chromosome (single copy) is lower than that of the disrupted locus from the multiple copy plasmid. Results from the genomic DNA samples of lanes 5 and 8 indicate that these two *A. tumefaciens* strains contain the GOI integrated in the chromosomal region (note that the intensity of the DNA bands is similar to that of the chromosomal locus). However, results from lanes 2–4, lanes 6–7, and lane 10 suggest that these *A. tumefaciens* strains do not have the GOI integrated (same pattern as lane 11). Interestingly, lane 9 shows that the *A. tumefaciens* strain has both a wild-type and a disrupted copy of the *pgl/picA* locus, indicating that this strain carries the entire Starting Construction as a cointegrate in its chromosome.

2. Materials

2.1. Growth and Preparation of *Agrobacterium*

1. YEP medium (per liter): 10 g peptone, 10 g yeast extract, 5 g NaCl. It is not necessary to adjust the pH. This medium is used to grow bacteria in liquid culture.
2. AB-sucrose minimum medium (14) is recommended for growth of *Agrobacterium* on solidified medium. When AB-glucose minimum medium is needed, replace the sucrose with glucose (10 g/L).

3. AB-sucrose minimal medium (per liter): add 5 g sucrose and 15 g Bacto Agar to 900 mL of distilled water. After medium is autoclaved, add 50 mL 20X AB salts, 50 mL 20X AB buffer, and the appropriate antibiotics.
4. 20X AB salts (per liter): 20 g NH_4Cl , 6 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3 g KCl, 0.26 g $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, and 0.05 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. Autoclave for 20 min.
5. 20X AB buffer (per liter): 78.6 g $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 23 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$. The pH of this buffer should be 7.0 without adjusting. However, if the buffer is acidic, adjust the pH using either KOH or NaOH. If the buffer is alkaline, use phosphoric acid to adjust the pH. Autoclave for 20 min.
6. Antibiotics for *Agrobacterium* selection: 50 $\mu\text{g/mL}$ carbenicillin in liquid medium and 100 $\mu\text{g/mL}$ in solid medium; 50 $\mu\text{g/mL}$ gentamicin; 50 $\mu\text{g/mL}$ kanamycin; 10 $\mu\text{g/mL}$ rifampicin; 50 $\mu\text{g/mL}$ spectinomycin in liquid medium and 100 $\mu\text{g/mL}$ in solid medium; 2 $\mu\text{g/mL}$ tetracycline in liquid medium and 4 $\mu\text{g/mL}$ in solid medium.
7. Grow the bacteria at 30°C.

2.2. Growth and Preparation of *E. coli*

1. LB medium (per liter): 10 g tryptone, 5 g yeast extract, 10 g NaCl. Use 10 N NaOH to adjust the pH to 7.0. For solid medium, add 15 g Bacto Agar before autoclaving.
2. Antibiotics for *E. coli* selection (final concentrations in mg/L): 100 mg/L ampicillin; 50 mg/L kanamycin; 50 mg/L gentamicin; 20 mg/L tetracycline; 100 mg/L spectinomycin.
3. Grow the bacteria at 30°C or 37°C.

2.3. Nucleic Acid Manipulations and *E. coli* Transformation

1. Restriction enzymes and modification enzymes.
2. T4 DNA ligase.
3. Competent *E. coli* cells: *E. coli* strains DH5 α or DH10B.
4. X-gal solution: 20 mg/mL X-gal in dimethyl formamide (DMF).

2.4. Bacterial Matings

1. Sterile 0.9% NaCl.
2. LB agar plates.
3. 30°C incubator.
4. *E. coli* strain E9 (containing pRK2013), required to mobilize plasmids between bacterial strains.
5. *E. coli* strain E4 (containing pPH1JI; see **Note 1**).
6. *Agrobacterium* strain containing the C58 chromosomal background (ACTT cat. no. 51350). *E. coli* strains E4, and E9 and *Agrobacterium* strains with C58 chromosomal background can be obtained from the author's laboratory.

2.5. Extraction of Total DNA from *Agrobacterium*

1. Bacterial washing buffer: 10 mM Tris-HCl, 10 mM EDTA, 300 mM NaCl, pH 8.0.

2. Bacterial lysis buffer: 50 mM Tris-HCl, 10 mM EDTA, 100 mM NaCl, 10 mg/mL lysozyme. Lysozyme should be freshly made up.
3. 5% Sarcosyl.
4. Tris-HCl saturated phenol/chloroform (1:1, v/v), pH 8.0.
5. Chloroform/isoamyl alcohol (24:1, v/v).
5. Absolute ethanol, 70% ethanol.
6. 3 M NaOAc, pH 5.2.
7. TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

3. Methods

3.1. Subcloning the GOI into the Integration Vector

1. Choose the appropriate restriction enzyme sites to use for insertion of the GOI into the integration vector (see **Note 2**).
2. Cut out your insert with appropriate restriction enzyme(s); elute the insert fragment from a gel if necessary. Follow the manufacturer's instructions.
3. Ligate your insert DNA to vector DNA and transform an *E. coli* host to obtain the Starting Construction.
4. Select and analyze the *E. coli* transformants. The *E. coli* colony with the correct Starting Construction will be used for the following experiment.

3.2. Introduction of Starting Construction into *Agrobacterium* Using Triparental Mating Conjugation (Also See Chapter 3)

1. Grow 5 mL liquid cultures of each bacterial strain overnight: *A. tumefaciens* containing the C58 chromosomal background (resistant to rifampicin), *E. coli* strain E9 (resistant to gentamicin), and the *E. coli* strain containing your Starting Construction (let's assume this strain is resistant to both ampicillin from the selectable marker near the GOI and tetracycline from the backbone of the integration vector) obtained in **Subheading 3.1.4**.
2. The next day, transfer 1 mL of each bacterial culture into an individual 1.5-mL microfuge tube. Centrifuge the cell pellets using the maximum microcentrifuge speed for 1 min.
3. Wash the cell pellets once with sterile 0.9% NaCl and resuspend the cells in 1 mL 0.9% NaCl.
4. Mix 0.2 mL of each bacterial solution in a 1.5-mL microfuge tube. Take 10 μ L of the bacterial mixture and spot on an LB agar plate.
5. Incubate the plate at 30°C overnight.
6. The next day, use 1 mL of sterile 0.9% NaCl to wash most of the bacteria from the mating plate. This is the undiluted bacterial conjugation mixture. Make a 10-fold serial dilution of this conjugation mixture with sterile 0.9% NaCl. Plate out 100 μ L of diluted (10^{-3} and 10^{-4} dilutions) conjugation mixture onto the selection medium containing the appropriate antibiotics (AB-sucrose medium containing rifampicin and carbenicillin).

7. Incubate the plates at 30°C for 2 to 4 d. Colonies appearing on these selection plates are potential transconjugants.
8. To confirm that *Agrobacterium* cells have received the Starting Construction, restreak several potential transconjugants grown from the method given in **Subheading 3.2.7.** on an AB-sucrose agar plate containing tetracycline to check antibiotic sensitivity (see **Note 3**). Pick one of the transconjugants that is resistant to tetracycline and continue the following experiment.

3.3. Eviction of the Starting Construction from *Agrobacterium*

1. Inoculate a single colony of the *A. tumefaciens* strain from the method given in **Subheading 3.2.8.** into 5-mL YEP liquid medium containing carbenicillin. Also inoculate a single colony of *E. coli* strain E4 in 5-mL LB liquid medium containing gentamicin.
2. Grow both cultures at 30°C overnight with shaking.
3. The next day, wash 1 mL of each culture once with sterile 0.9% NaCl in a sterile microcentrifuge tube; then resuspend the cell pellets in 1 mL of 0.9% NaCl.
4. Mix 0.2 mL of *Agrobacterium* cells with 0.2 mL of *E. coli* strain E4 in a tube. Take out 10 μ L of mixture and spot on an LB agar plate.
5. Incubate the plate at 30°C overnight. The next day, use 1 mL of sterile 0.9% NaCl to suspend the conjugation mixture. Make 10-fold serial dilutions in 0.9% NaCl and plate 100 μ L of each dilution (10^{-2} , 10^{-3} , and 10^{-4} dilutions) on selection medium (AB-sucrose agar medium containing carbenicillin and gentamicin) to screen for cells that have the GOI and selection marker integrated onto the chromosomal *pgl/picA* locus through homologous recombination.
6. Incubate the plate at 30°C for 2 to 3 d. Colonies appearing on selection medium potentially contain the GOI integrated into the *Agrobacterium* chromosome.
7. Streak several colonies from the previous step on plates containing AB-sucrose agar medium with either carbenicillin and gentamicin, or tetracycline, respectively. Only keep the strains that are resistant to both carbenicillin and gentamicin and are sensitive to tetracycline.

When pE1962 or pE1963 is used:

8. Inoculate a single colony of the *A. tumefaciens* strain from the method given in **Subheading 3.2.8.** into 5 mL YEP liquid medium containing carbenicillin and culture the bacteria at 30°C overnight.
9. Centrifuge 1 mL of bacterial culture and wash once in 0.9% NaCl. Resuspend the cell pellet in 1 mL of 0.9% NaCl. Make 10-fold serial dilutions of the cell suspension. Plate out 100 μ L of each dilution (use 10^{-6} and 10^{-7} dilutions) on an AB-glucose agar plate containing carbenicillin (or the antibiotic for the selection marker that is linked to the GOI). Incubate the plates for 2 to 4 d at 30°C.
10. Streak the colonies obtained from the method given in **Subheading 3.3.9.** on an AB-sucrose agar plate and an AB-glucose agar plate that have the same antibiotic as in the previous step. Let the bacteria grow for 1 to 2 d. Keep the colonies that grow on glucose- but not on sucrose-containing medium for the following experiment.

3.4. Confirmation of the Integration of the GOI

3.4.1. Extraction of Total DNA from *Agrobacterium* (Also See Chapter 5)

1. Culture several *Agrobacterium* colonies obtained from **Subheadings 3.3.7.** or **Subheadings 3.3.10.** in 5 mL YEP liquid medium at 30°C overnight.
2. The next day, centrifuge 1 mL of bacterial culture and wash the cell pellets once with bacterial washing buffer. Resuspend the cells in 400 μ L of bacterial lysis buffer. Add 133 μ L of 5% sarcosyl and incubate the tube at 65°C for 20 min.
3. Add 500 μ L of Tris-HCl-saturated phenol/chloroform to the tube and vortex vigorously for 1 min. Centrifuge the tube at the maximum speed for 10 min in a microcentrifuge.
4. Transfer the upper aqueous solution to a new microcentrifuge tube. If some of the protein interphase is transferred to the new tube, repeat **step 3** one more time. Add 1 mL of chloroform/isoamyl alcohol to the tube, and vortex for 30 s. Centrifuge for 5 min and then transfer the upper aqueous solution to a new tube.
5. Add 1 mL of absolute ethanol and 50 μ L of 3 M NaOAc, pH 5.2, to the tube to precipitate the DNA. Incubate the tube at -20°C for 30 min.
6. Centrifuge the DNA pellet in a microcentrifuge at the maximum speed for 10 min. Wash the pellet once with 70% ethanol and dry the pellet at room temperature for 10 min. Use 200 μ L of TE buffer to dissolve the DNA pellet (see **Note 4**).
7. Measure the DNA concentration using a spectrophotometer.

3.4.2. DNA Digestion

1. Digest to completion 2 μ g each of several samples of total DNA obtained from the method given in **Subheading 3.3.7.** with the restriction enzyme *Eco*RI (see **Note 5**). Digest 200 ng of the integration vector with the same enzyme.
2. Subject the digested DNA samples and DNA molecular weight markers to electrophoresis (1 μ g of digested total DNA from *Agrobacterium* and 50 ng of digested integration vector DNA) through a 0.7% agarose gel (see **Note 6**).
3. Take a picture of the ethidium bromide-stained gel with a ruler on the side of gel to mark the position of the DNA bands.
4. Transfer the DNA onto a nylon membrane by following a standard DNA blotting protocol.
5. Perform hybridization by using the 3.15-kbp *Eco*RI fragment containing the *pgl/picA* locus from plasmid pE578 (**Fig. 1**) as a probe.

4. Notes

1. *E. coli* strain E618 can be used when your *Agrobacterium* strain is already resistant to gentamicin. This strain contains pVK102, which carries kanamycin and tetracycline resistance genes.
2. If one wants to place both a GOI and a selectable marker into the integration vector to make the Starting Construction, one can use pE1770 or pE1773. If one wants to clone a GOI into an integration vector that already contains a selectable

- marker, one can use pE1931 or pE1963. If one is using the sucrose counterselection method, one can use pE1963 or pE1962 (**Fig. 1**).
3. Alternatively, one can extract plasmid DNA from the *Agrobacterium* transconjugant and retransform *E. coli* with this plasmid DNA. If the new *E. coli* transformants are now resistant to both markers (in this case, they are ampicillin and tetracycline) from the Starting Construction, this suggests that this *Agrobacterium* transconjugant does contain the Starting Construction.
 4. It takes a long time to dissolve bacterial genomic DNA at this concentration. One may wish to keep the tube at 37°C for about 30 min to help dissolve the DNA. Alternatively, one may use more TE buffer to dissolve the DNA. *Do not vortex the DNA*; it will shear.
 5. After digesting the total DNA for an hour or so, take out 500 ng of DNA and load onto an agarose gel to check for completion of digestion. If digestion is incomplete, add more enzymes and continue the digestion. Proceed to the next step only when the digestion is complete.
 6. Use a gel at least 15 cm in length. During electrophoresis, use a low voltage (<40 V) and run the gel for a longer time (>5 h) to ensure good separation of the DNA bands. Do not run small DNA fragments (such as a 300-bp fragment) off the bottom of the gel.

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Nucleic Acid Extraction from *Agrobacterium* Strains

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Summary

Agrobacterium is routinely used as a tool for moving genetic constructs into plant cells. The successful use of *Agrobacterium* as a tool for the genetic engineering of plant cells often requires the manipulation and analysis of nucleic acids present in recombinant *Agrobacterium* strains. Here we present dependable methods for the isolation of genomic (total) DNA, mega-plasmid DNA, shuttle or binary plasmid DNA, and RNA. In addition, we provide a simple method for the electronic transfer of shuttle plasmids from *Agrobacterium* to *E. coli* for use when their low copy number in *Agrobacterium* impedes plasmid isolation from that strain.

Key Words: Nucleic acids; RNA; genomic DNA; mega-plasmid; pTi; pRi; shuttle plasmid; binary plasmid.

1. Introduction

Agrobacterium's capacity to introduce and stably integrate almost any DNA into a variety of plant cell genomes makes this organism the tool of choice for the production and study of recombinant plants. Often, circumstances arise in which it is advantageous to manipulate, analyze, or confirm the genetic material carried by the bacterial engineer.

The isolation of genomic (including both chromosome and plasmid) DNA from *Agrobacterium* is an essential step for a number of procedures including determination of plasmid copy number, confirmation of DNA integrated during strain construction, or provision of polymerase chain reaction (PCR) template. Pure RNA is needed if one wishes to analyze gene transcription levels in the bacteria through Northern blot or reverse transcription (RT)PCR analysis. Purification of pTi and pRi allows the manipulation and analysis of these mega-plasmids and facilitates their transfer (through electroporation) to strains that do not carry their own version of the plasmid. Gene libraries maintained in

Agrobacterium may require the isolation of binary plasmids from the bacteria to determine library quality or to identify genes that confer a desired plant phenotype. In addition, plasmid isolation may be useful in confirming the genotype of an *Agrobacterium* strain to be used in recombinant plant work.

Isolation of plasmid DNA from *Agrobacterium* is slightly more complex than plasmid isolation from *E. coli*. Miniprep versions of the process for both genera commonly involve alkaline lysis and neutralization with salts that also assist in an alcohol-based precipitation process. *Agrobacterium* cells take somewhat longer than *E. coli* to lyse, and obtaining clean plasmid DNA from *Agrobacterium* usually necessitates a phenol extraction step. The yield of plasmid DNA will depend strongly on copy number. Because the copy number of many *E. coli*/*Agrobacterium* shuttle vectors is lower in *Agrobacterium* than in *E. coli*, it is sometimes beneficial to transfer the plasmid from *Agrobacterium* to *E. coli*, in which it can be obtained in amounts sufficient for analysis and manipulation. Here we present methods for the isolation and purification of genomic DNA, mega-plasmid DNA, binary and shuttle plasmid DNA, and RNA, as well as an electronic method for the transfer of shuttle plasmid DNA from *Agrobacterium* to *E. coli*.

2. Materials

2.1. Bacterial Media

1. LB: 10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract. Dissolve ingredients in water. Bring volume to 1 L. If making plate medium, add 15 g/L agar. Autoclave.
2. MG/L: 5 g/L tryptone, 2.5 g/L yeast extract, 5 g/L NaCl, 5 g/L mannitol, 0.1 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 g/L K_2HPO_4 , 1.2 g/L L-glutamate, thiamine (10% solution, filter-sterilized). Dissolve all ingredients except thiamine in 900 mL water. Check that pH is close to 7.0. Bring volume to 1 L. If making plate medium add 15 g/L agar. Autoclave. When media cools to 60°C or below, add 120 $\mu\text{L/L}$ of the thiamine solution.
3. YEB: 5 g/L tryptone, 1 g/L yeast extract, 5 g/L nutrient broth, 5 g/L sucrose, 0.49 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Dissolve ingredients in water. Adjust the pH to 7.2 and bring volume to 1 L. Autoclave.

2.2. Solutions and Supplies

2.2.1. Genomic (Total DNA) Isolation

1. Cell wash solution: 50 mM Tris-HCl (pH 8.0), 20 mM EDTA, 0.5 M NaCl, 0.05% sarkosyl.
2. 10X TE buffer: 10 mM EDTA, 100 mM Tris-HCl (pH 8.0).
3. Lysozyme solution (10 mg/mL).
4. RNase A: DNase-free RNase (Roche Applied Science). Alternatively, boil a solution of 10 mg/mL RNase A in TE for 10 min to inactivate DNases. Cool slowly to room temperature. Store at -20°C.

5. Lysis buffer: 1% sodium dodecyl sulfate (SDS), 200 µg/mL proteinase K in 10X TE.
6. Phenol buffered with 1.0 M Tris-HCl (pH 8.0) until the aqueous phase has a pH between 7.6 and 7.8. Alternatively, purchase buffered phenol (Pierce, cat. no. 17912).
7. 24:1 Solution of chloroform/isoamyl alcohol.
8. Ethanol: absolute and 70%.
9. Spectrophotometer with UV capability.
10. Quartz spectrophotometer cuvet with 1-cm pathlength.

2.2.2. Isolation of Mega-Plasmid (*pTi*, *pRi*) DNA

1. Cell wash solution: 50 mM Tris-HCl (pH 8.0), 20 mM EDTA, 0.5 M NaCl, 0.05% sarkosyl.
2. Cell resuspension solution: 25 mM Tris-HCl (pH 8.0), 10 mM EDTA, 50 mM glucose, 2 mg/mL lysozyme.
3. Lysis solution: 0.2 N NaOH, 1% SDS. Prepare fresh.
4. 2 M Tris-HCl (pH 7.0).
5. 5 M NaCl.
6. Phenol buffered with 1.0 M Tris-HCl (pH 8.0) until aqueous phase has a pH between 7.6 and 7.8. Alternatively, purchase buffered phenol (Pierce, cat. no. 17912).
7. Chloroform/isoamyl alcohol (24:1, v:v).
8. Ethanol: absolute and 70%.

2.2.3. Isolation of Shuttle and Binary Plasmid DNA

1. GETL solution: 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0), 0.4 mg/mL lysozyme.
2. Lysis solution: 0.2 N NaOH, 1% SDS. Prepare fresh.
3. 3 M Potassium acetate, pH 4.8.
4. Chloroform/isoamyl alcohol (24:1).
5. Phenol/chloroform/isoamyl alcohol (25:24:1).
6. Isopropanol.
7. 80% Ethanol.
8. TE or sterile distilled-deionized water.
9. RNase A: DNase-free RNase (Roche Applied Science). Alternatively, boil a solution of 10 mg/mL RNase A in sterile water for 10 min to inactivate DNases. Cool slowly to room temperature. Store at -20°C.

2.2.4. Electrotransfer of Plasmid DNA from *Agrobacterium* to *E. coli*

1. Electro-competent *E. coli* cloning strain (see **Note 1**).
2. Electroporation apparatus.
3. Two 0.2-cm-gap electroporation cuvetts.
4. Sterile distilled-deionized water.
5. Sterile Pasteur pipets and rubber bulb.
6. Refrigerated microcentrifuge.

2.2.5. RNA Isolation

1. Glassware for making and storage of solutions. Bake overnight at 180°C to inactivate RNases.
2. Sterile RNase-free pipet tips and microcentrifuge tubes.
3. Diethylpyrocarbonate (DEPC)-treated water: Add DEPC (0.1%) to distilled-deionized water and mix vigorously. Let set for at least 2 h. Autoclave for 1 h.
4. 3 M NaOAc (pH 5.5) made with DEPC-treated water.
5. 500 mM EDTA (pH 8.0) made with DEPC-treated water.
6. Cell resuspension solution: 20 mM NaOAc, 1 mM EDTA. Make this with the above DEPC-treated components and autoclave 1 h.
7. Acidic SDS-phenol solution: equilibrate phenol with 20 mM NaOAc (pH 5.5). Add SDS to 1%.
8. 1:1 (v/v) acidic phenol/dichloromethane (see **Note 2**).
9. 1:1 (v/v) dichloromethane/10 mM Tris-HCl (pH 7.0) (see **Note 2**).
10. Ice-cold absolute ethanol.
11. 70% Ethanol prepared with DEPC-treated water.

3. Methods

3.1. Isolation of Genomic (Total) DNA from *Agrobacterium*

A similar method starting with 500 mL of cells is described by Charles and Nester (**1**).

1. Inoculate 4.0 mL growth medium, e.g., MG/L or YEB, with the *Agrobacterium* strain. Incubate the cells with shaking to aerate overnight at 25–28°C.
2. Transfer 1.5 mL of cell culture to a 1.5-mL microcentrifuge tube and spin at 12,000g for 2 min to pellet the cells. Remove the supernatant, and repeat with another 1.5 mL of the culture to concentrate the cells in the microcentrifuge tube. Wash the cell pellet with 700 µL cell wash solution.
3. Resuspend the cells in 100 µL 10X TE buffer with repeated pipeting. Add 20 µL lysozyme solution, 2 µL RNase and incubate for 15–30 min at 37 °C.
4. Add 450 µL lysis buffer. Mix and incubate for 1 h at 50°C.
5. Extract the cell solution with 500 µL buffered phenol (see **Note 3**). Centrifuge for 5 min at 12,000g at room temperature to separate organic and aqueous layers.
6. Transfer the upper aqueous phase to a clean tube (see **Note 4**). Extract with an equal volume of a 1:1 mixture of buffered phenol and the 24:1 chloroform/isoamyl alcohol solution. Again, mix well, and centrifuge for 5 min. Transfer the aqueous phase to a clean tube and repeat the extraction until the interface between the two layers is clean.
7. A final extraction of the aqueous phase with the 24:1 chloroform/isoamyl alcohol is done to remove traces of phenol. Mix gently, centrifuge for 5 min, and remove the upper layer to a clean tube.
8. Add 2 vol of ice-cold absolute ethanol to the tube and mix well until the DNA precipitates (see **Note 5**). Centrifuge at 12,000g for 10 min at 4°C. Discard the

supernatant and wash with 70% ethanol. Drain well and let the pellet dry on the benchtop for about 30 min.

9. Resuspend the DNA in 1X TE (25–100 μ L).
10. The amount and purity of DNA can be assessed by taking optical density readings in the UV range. Place 10 μ L of your genomic DNA solution into 990 μ L 1X TE. Set a UV spectrophotometer to read optical density at 230, 260, and 280. Blank the spectrophotometer with TE in a quartz cuvet. Using the same cuvet, take the readings for your DNA solution. Calculate the amount of DNA as follows (see **Note 6**):

$$(\text{OD}_{260} \times 50 \text{ } \mu\text{g/mL}) / 10 \text{ } \mu\text{L DNA} = \mu\text{g DNA}/\mu\text{L}$$

An $\text{OD}_{260}/\text{OD}_{280}$ ratio of approx 1.8 indicates clean DNA, as proteins absorb at 280 and will lower that ratio. However, an $\text{OD}_{260}/\text{OD}_{230}$ ratio over 2.0 is a more sensitive measurement for the determination of nucleic acid purity as several substances including protein and phenols absorb light strongly at a wavelength of 230.

3.2. Isolation of *pTi* or *pRi* Plasmids

This method for isolating mega-plasmids is based on one detailed by Hayman and Farrand (2).

1. Grow *Agrobacterium* to late exponential phase in 200 mL LB. Chill the culture on ice. Pellet the cells by centrifuging at 10,000g for 15 min at 4°C.
2. Resuspend the cell pellet in 15 mL cold cell wash solution. Repeat centrifugation and discard the supernatant.
3. Resuspend the cells thoroughly in 15 mL cold cell resuspension solution with repeated pipeting. Set on ice for 5 to 10 min.
4. Add 30 mL freshly made lysis solution and mix gently by inverting the tube several times. Let set at room temperature for 10 min.
5. Add 7.5 mL of 2 M Tris-HCl (pH 7.0). Mix tube contents gently by inverting the tube.
6. Add 7.5 mL 5 M NaCl and mix well with tube inversion. Let the solution stand at room temperature for 20 min.
7. Add an equal volume of buffered phenol and an additional 1.8 mL of 5 M NaCl. Mix by repeated inversion of the tube (see **Note 3**). Centrifuge at 10,000g for 10 min at 4°C. Transfer the aqueous phase to a clean centrifuge bottle. Repeat the extraction with a 25:24:1 mix of phenol/chloroform/isoamyl alcohol until the interface between the aqueous and organic phases is clean. Do a final extraction with 24:1 chloroform/isoamyl alcohol.
8. Precipitate DNA with the addition of 2 vol of ice-cold ethanol. Place at –20°C overnight.
9. Collect the DNA by centrifuging at 10,000g for 20 min at 4°C. Carefully decant the supernatant. Wash the DNA pellets with 70% ethanol, repeating the centrifugation step. Remove the ethanol, and air-dry the DNA. Resuspend in 25 μ L TE (see **Note 7**).

3.3. Alkaline Lysis Plasmid Miniprep for Shuttle and Binary Plasmids

With the exception of the added phenol/chloroform extraction, this method is similar to several mini-prep protocols including one described by Brent and Irwin (3).

1. Inoculate 4.0 mL rich liquid medium containing appropriate antibiotics for plasmid maintenance with your *Agrobacterium* strain. Incubate with shaking overnight at 25 to 28°C to early stationary phase (see **Note 8**).
2. Transfer 1.5 mL of cell culture to a 1.5-mL microcentrifuge tube and spin at 10,000g for 2 min to pellet cells. Remove the supernatant and repeat with another 1.5 mL of the culture to concentrate the cells in the microcentrifuge tube.
3. Resuspend the cell pellet in 150 μ L GETL. Let set at room temperature for 5 min.
4. Add 300 μ L lysis solution. Invert tube several times to gently mix. Set on ice for 5 min.
5. Add 250 μ L 3 M potassium acetate, pH 4.8. Invert several times to mix. Set the tube on ice for 10 min and occasionally mix.
6. Pellet cell debris by centrifuging at high speed for 10 min. Transfer the supernatant to a clean tube.
7. Add 2 μ L of RNase A (10 mg/mL). Incubate at room temperature for 20 min.
8. Add an equal volume (700 μ L) of phenol/chloroform/isoamyl alcohol (25:24:1). Mix well for about 5 min (see **Note 3**).
9. Centrifuge at 12,000g for 5 min and remove the upper aqueous layer to a clean tube (see **Note 4**).
10. Repeat the extraction with 24:1 chloroform/isoamyl alcohol to remove traces of phenol. Centrifuge at high speed for 5 min and remove upper layer to a clean tube.
11. Precipitate plasmid by adding an equal volume of isopropanol. Place at -20°C for at least 30 min. Centrifuge at 12,000g for 20 min and carefully pour off the supernatant.
12. Wash the pellet once or twice with 1 mL 70% ethanol, spinning for 5 min after each addition.
13. Drain the pellet well and let dry on benchtop for 20 or 30 min. Resuspend in 20 μ L TE or sterile distilled-deionized water. Plasmid can be stored long term at -20 or -70°C.

3.4. Electronic Transfer of Plasmid DNA from *Agrobacterium* to *E. coli*

The development of this protocol was inspired by an electronic direct transfer method described by Vujaklija and Davies (4).

1. Prepare electro-competent *E. coli* recipient cells (5) but reduce the final volume to concentrate the cells further (see **Note 1**).
2. Grow the *Agrobacterium* donor strain to stationary phase in 4 mL rich medium with antibiotics to maintain the plasmid you wish to transfer to *E. coli*.

3. Transfer 1.5 mL of cell culture to a microcentrifuge tube and spin at 10,000g for 2 min to pellet the cells. Remove the supernatant and repeat with another 1.5 mL of the culture to concentrate the cells in the tube.
4. Resuspend the *Agrobacterium* cells in 800 μ L ice-cold sterile distilled-deionized water. Pellet the cells, remove the supernatant, and repeat (see **Note 9**).
5. Resuspend *Agrobacterium* cells in 80 μ L ice-cold water and freeze at -80°C (see **Note 10**).
6. Prepare for the plasmid transfer in the following manner: (1) thaw both the *Agrobacterium* cells and the electro-competent *E. coli* cells on ice; (2) chill two 0.2-cm electro-cuvets and two 1.5-mL microcentrifuge tubes on ice; (3) prepare a glass culture tube (16 $\times$ 125 mm) with 2 mL LB (no antibiotics) and have an additional small amount of LB (300 μ L is sufficient) ready; (4) set the electro-poration apparatus to 12.5 kV, 25 μ Fd, and 100 Ω .
7. Transfer the *Agrobacterium* cells to one of the chilled cuvettes. Give the cells two electric pulses within 3–5 s (see **Note 11**).
8. Immediately transfer the pulsed *Agrobacterium* cells to one of the chilled microcentrifuge tubes and centrifuge at 12,000g for 5 min at 4°C . Avoiding cells and debris, transfer the supernatant to the second chilled tube (see **Note 12**).
9. Mix the electrocompetent *E. coli* cells with the recovered supernatant and place in the second chilled cuvet. Deliver one electric pulse to the mixture at 2.5 kV, 25 Fd, and 200 Ω . Add 300 μ L LB to the cuvet and transfer the cells to a culture tube containing LB with a sterile Pasteur pipet.
10. Incubate the *E. coli* cells at 37°C with shaking for several hours or overnight (see **Note 13**).
11. With two successive spins, centrifuge the cells to concentrate them in a microcentrifuge tube. Resuspend cells in 500 μ L LB with antibiotic for selection of the plasmid. Make serial dilutions (10^{-1} , 10^{-2}) in the same media. Plate 100 μ L from each of the three tubes on selective medium and incubate overnight at 37°C (see **Note 14**).

3.5. RNA Isolation

This method closely follows one described by Liu and Binns (6).

1. Grow the *Agrobacterium* strain in medium that allows expression of the genes whose RNA is to be analyzed (see **Note 15**).
2. Harvest the cells by centrifuging at 10,000g for 10 min. Pour off the supernatant and resuspend cells in 1.6 mL of the cell resuspension solution (see **Note 16**).
3. Add to 1 mL of prewarmed (65°C) acidic SDS-phenol solution. Mix well and incubate at (65°C) for 7 min (see **Notes 3 and 17**).
4. Centrifuge at 12,000g for 5 min in a microcentrifuge at room temperature. Remove the top aqueous layer to a clean tube. Repeat the phenol extraction until the interface between the two layers is nearly clean.
5. Add 1 mL of 1:1 phenol/dichloromethane to the aqueous phase (see **Notes 2 and 17**). Mix well and centrifuge at 12,000g for 5 min at room temperature. Remove the aqueous phase to a clean tube.

6. Repeat the extraction process with 1 mL of the Tris-equilibrated dichloromethane. Remove the aqueous phase to a clean tube.
7. Add 2.5 vol of absolute ethanol to the aqueous phase and spin at 12,000g for 10 min in a microcentrifuge at 4°C to precipitate the RNA.
8. Wash the pellet with 70% ethanol and air-dry for 10–15 min. Resuspend in 25 to 50 μ L of DEPC-treated water (see **Note 18**).

4. Notes

1. This method depends on capturing a relatively small amount of plasmid carried in the *Agrobacterium* cells. Thus, the success of the method relies on a very dense resuspension of *E. coli* recipient cells. If you have electro-competent *E. coli* stored in your freezer, you can concentrate the cells from two or three tubes. Gently thaw the cells, and centrifuge in a microcentrifuge at low speed (5,000g). Remove the supernatant with a pipet tip and combine the cells into one tube.
2. The use of dichloromethane is favored for the extraction process as it provides a more defined interface between the organic and aqueous phases. However, a 24:1 solution of chloroform/isoamyl alcohol may be used instead of the dichloromethane.
3. Thorough mixing of the cell solution and phenol is essential for efficient extraction of proteins and lipids. To do this, invert tube repeatedly for 3 to 5 min. Also, note that phenol and chloroform and their fumes are toxic. Wear appropriate protective clothing (lab coat, goggles, and gloves) and work in a ventilated chemical hood. It is convenient to have a microcentrifuge placed in the hood for use during phenol/chloroform extractions.
4. The aqueous upper phase will contain the DNA, but note that high salt concentrations can reverse the positions of the organic and aqueous phases. The presence of chloroform and isoamyl alcohol in the phenol extraction provides a more defined boundary between the two phases, facilitating removal of the top layer.
5. The translucent nature of clean DNA can make it difficult to see small amounts. Also, if the DNA concentration is very dilute, it may not be possible to see a precipitate following the addition of ethanol. Low yields are usually the result of insufficient mixing in **step 5 (Subheading 3.1.)**. The precipitation of dilute DNA can be increased by adding 2.5 μ L 3M NaOAc, pH 5.5, 1 μ L glycogen (20 mg/mL) per 100 μ L DNA solution, and 2 vol of ethanol. Place at -20°C for a few hours before precipitating and washing as described in **step 8 (Subheading 3.1.)**.
6. The expected yield of genomic DNA is between 15 and 50 μ g for 3 mL of a stationary phase cell culture.
7. This method will provide sufficient mega-plasmid DNA for use as a PCR template or for transferring the plasmid to a second *Agrobacterium* strain through electroporation.
8. If your colonies are very small, inoculation with a single colony and overnight growth may not provide sufficient cells for this method. It is tempting to use multiple colonies in the inoculation step. However, this approach will occasionally create confusion in subsequent procedures that make use of the plasmid DNA. It is better technique to lengthen the growth period as needed.

9. A thorough washing of the *Agrobacterium* donor cells is important. Salts that remain in the solution recovered from postshocked *Agrobacterium* may cause electrical arcing during the transfer of plasmid to *E. coli*. This will increase cell death and reduce the chances of obtaining transformants.
10. The freeze/thaw step weakens the cell wall and increases the exit of plasmid out of *Agrobacterium* in the next step. However, this is also a good stopping point, and the resuspended cells can be left at -80°C until it is convenient to continue with the procedure.
11. The time constant for the pulse will be approximately 1.5 to 1.8 ms. The electric shock works, as in electroporation, to open pores in the cell. In this step, the method depends on plasmid DNA leaving cells rather than entering them.
12. It is important to proceed quickly after delivering the electronic pulses to the *Agrobacterium* cells. Apparently, nucleases are activated by the shock. In any case, plasmid DNA in the recovered supernatant is unstable and should be transferred to *E. coli* without delay.
13. The efficiency of this method is significantly lower than directly electroporating purified plasmid into *E. coli*, but it is also much simpler because it avoids the process of first purifying the plasmid from *Agrobacterium*. The number of transformants recovered varies with plasmid size and the *Agrobacterium* copy number, which may be significantly lower than the copy number in *E. coli*. A 15-kb plasmid of 20 to 40 copies per *Agrobacterium* provides a total of 6000 to 7000 transformants with this method. (Not all are plated.) With a 36-kb plasmid of five to eight copies per cell in *Agrobacterium*, the method was 10 to 50 times less efficient but still very serviceable. Increasing the incubation period for the transformed *E. coli* cells (before plating) is helpful when one is transferring low-copy plasmids. In this case, a selective antibiotic should be added to the liquid *E. coli* culture after the first 2 or 3 h of incubation.
14. The electronic transfer method facilitates the construction of *E. coli*/*Agrobacterium* shuttle vectors and the recovery of plasmids conferring a phenotype that can be selected or screened for in *Agrobacterium*. For example, a ligation reaction intended to add an origin of replication that will function in *Agrobacterium* to an *E. coli* replicon can be directly electroporated into *Agrobacterium* with antibiotic selection derived from the *E. coli* replicon. Transformants will necessarily carry a plasmid with origins of replication for both bacteria. The electronic method is then used to transfer the plasmid back to *E. coli* for confirmation through restriction digests and any further construction steps.
15. The growth medium and the amount of cell culture prepared will depend on the expression pattern for the gene(s) of interest. In addition, if expression (or RNA stability) is influenced by growth phase, you will need to consider that when determining the appropriate time to harvest the cells. The amount of cells required for this method is equivalent to 10 mL with an OD_{600} between 1.2 and 1.6 per mL. If you harvest cells at a lower optical density, increase the amount of starting culture to give you an equivalent quantity of cells.

16. At this point, it is convenient to divide your resuspended cells into two or more microcentrifuge tubes in order to accommodate the extraction process that follows. In the following steps, divide the specified volumes of phenol or dichloromethane equally among the tubes.
17. Dichloromethane, like phenol and chloroform, is toxic, and appropriate measures should be taken. Place a heating block set to 65°C in the chemical hood for use during the RNA extraction procedure.
18. Quantify the yield of RNA by measuring the OD₂₆₀. An absorbance of 1 U at OD₂₆₀ is equivalent to 40 µg/mL RNA.

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Agrobacterium Virulence Gene Induction

Stanton B. Gelvin

Summary

The ability of *Agrobacterium* to transform plants and other organisms is under highly regulated genetic control. Two Virulence (Vir) proteins, VirA and VirG, function as a two-component regulatory system to sense particular phenolic compounds synthesized by wounded plant tissues. Induction by these phenolic compounds, in the presence of certain neutral or acid sugars, results in activation of other *vir* genes, leading to the processing of T-DNA from the Ti-plasmid and transfer of T-DNA to recipient host cells. Many plant, and most nonplant, species do not provide sufficient quantities of the correct phenolic compounds to permit efficient *Agrobacterium*-mediated genetic transformation to occur. In order to transform these species, phenolic inducing compounds must be added to *agrobacteria* before and/or during cocultivation of recipient cells with the bacteria. This chapter discusses conditions for efficient induction of *Agrobacterium* virulence genes by phenolic compounds.

Key Words: *Agrobacterium tumefaciens*; virulence genes; plant genetic transformation; plant genetic engineering; *vir* gene induction; phenolic compounds.

1. Introduction

Agrobacterium tumefaciens genetically transforms plants and other organisms by transferring a portion of a large Ti (tumor inducing)-plasmid, the T (transferred)-DNA, from the bacterium to the host (reviewed in [refs. 1](#) and [2](#)). Proteins required for processing of the T-DNA from the Ti-plasmid, export of the T-DNA from the bacterium, and “guiding” the T-DNA to the nucleus once inside the eukaryotic host cell are encoded by genes residing in the virulence (*vir*) region of the Ti-plasmid. Many transformation-associated events that occur within the bacterium are under tight genetic regulation. These events are directed by proteins encoded by the “*vir* regulon”, a group of genes that respond to phenolic molecules synthesized by wounded (and therefore susceptible) plant cells.

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The “sensor” for the *vir*-inducing phenolics is the VirA protein, a member of a two-component genetic regulatory system (reviewed in **ref. 3**). VirA localizes to the bacterial periplasmic membrane. In the presence of specific phenolic molecules, and in the presence of specific neutral or acidic sugars that interact with the ChvE protein (**4,5**), VirA autophosphorylates and subsequently transfers the phosphate to an inactive “response regulator” protein, VirG (**6,7**). Phosphorylation “activates” VirG, which then binds to “vir box” sequences within the promoters of *vir* regulon genes, stimulating their transcription (**8,9**).

The VirA/VirG-controlled *vir* regulon is usually induced by specific classes of phenolic molecules. Although numerous molecules that can stimulate *vir* gene induction have been described (including vanillin, methyl sinapate, methyl ferulate, methyl syringate, syringaldehyde, coniferyl alcohol, 2',4',4'-trihydroxy-3-methoxychalcone, and 2',4',4'-trihydroxy-3,5-dimethoxychalcone), acetosyringone (3,5-methoxy-4-hydroxyacetophenone [AS]) has become the favored molecule to use for inducing the *vir* genes of *Agrobacterium* (**10–17**). AS from tobacco exudates was the first *vir* inducer described (**10**) and because of its low cost and commercial availability has become the compound of choice.

Over the years, numerous articles have been published describing optimal conditions for *Agrobacterium vir* gene induction by AS. Although details of the results may differ among these various reports, a general consensus exists for many of these conditions. *Vir* gene induction is maximal at acid pH (~5.2–6.0); there is little induction at neutral pH (**18–20**). Therefore, it is useless to add AS to the “rich media” (pH 7.0) frequently utilized to grow *Agrobacterium* vegetatively (this is a common mistake.) Rich medium, even when adjusted to pH 5.6, also is a poor *vir* induction medium (**20**); conditions commonly used for *vir* induction utilize a “minimal medium” base. The temperature optimum for *vir* gene induction (~25°C) is generally lower than that optimal for vegetative growth of *Agrobacterium* (28–30°C; **21,22**). In addition, certain “coinducing” sugars such as glucose (which, in the presence of ChvE protein enhance *vir* gene induction by low concentrations of AS) are frequently substituted by sucrose in vegetative growth medium. However, sucrose is not an effective *vir* gene “coinducer” (**23**).

Taking into consideration the issues discussed above, we have developed an effective *vir* gene induction protocol that has permitted us to investigate the early events of T-DNA transfer from *Agrobacterium* to plant cells. These studies required a rapid and synchronous infection of a high percentage of tobacco, maize, or *Arabidopsis* suspension culture cells; thus, induced *Agrobacterium* cells could be considered “spring-loaded”, with the T-DNA processed from the Ti-plasmid and the type IV secretion system (encoded by the 11 VirB pro-

teins and VirD4) assembled and ready to transfer T-DNA and Vir proteins as soon as the bacteria attached to plant cells (24,25). The protocol described here can be used to induce the *vir* genes of any *Agrobacterium* strain.

Numerous *Agrobacterium* strains have been developed that contain a reporter gene, such as *lacZ*, inserted into a *vir* gene (26). These strains can be used to monitor *vir* gene induction by monitoring induction of β -galactosidase activity and can therefore serve as a check on the *vir* gene induction medium and AS. One such useful strain is *A. tumefaciens* A348::mx358 (27). This strain carries a Tn3-HoHo1 (a transposon containing a *lacZ* gene) insertion in the *virE* operon. This strain displays very low β -galactosidase activity in vegetatively growing bacteria. However, upon induction of the *virE*::*lacZ* fusion gene, there is a large increase in β -galactosidase activity.

Although *vir* gene induction by phenolic compounds is normally a prerequisite for transformation, the reader should note that several mutant *Agrobacterium* strains, containing constitutively “active” VirA or VirG proteins, have been identified (28–30). In some circumstances, these “induction-independent” strains have been shown to elicit higher transformation frequencies than do their wild-type counterparts (31).

2. Materials

2.1. *Agrobacterium tumefaciens* Strains

1. Any *Agrobacterium* strain of interest harboring a *lacZ* gene.
2. *A. tumefaciens* A348::mx358 (27). This strain contains a Tn3-HoHo1 insertion into the *virE* locus of the Ti-plasmid pTiA6.

2.2. Vegetative Growth of *Agrobacterium*

1. Sterile 0.9% NaCl.
2. YEP medium (per liter): 10 g peptone, 10 g yeast extract, 5 g NaCl. If the medium is to be solidified in Petri plates, add 15 g Bacto Agar before autoclaving. It is not necessary to adjust the pH.
3. AB-sucrose medium: combine 50 mL sterile 20X AB buffer and 50 mL sterile 20X AB salts with 900 mL sterile sucrose-water (final concentration of sucrose in 1 L is 0.5%).
 - a. 20X AB buffer (per liter): 60 g K_2HPO_4 , 20 g NaH_2PO_4 , pH to 7.0 using either KOH or H_3PO_4 , as required, before autoclaving.
 - b. 20X AB salts (per liter): 20 g NH_4Cl , 6 g $MgSO_4 \cdot 7H_2O$, 3 g KCl, 0.2 g $CaCl_2$, 50 mg $FeSO_4 \cdot 7H_2O$.
4. Spectrophotometer.
5. Centrifuge and sterile capped centrifuge tubes.

2.3. Induction of *Agrobacterium vir* Genes

1. Induction medium: 1X AB salts, 2 mM phosphate buffer (pH 5.6), 50 mM 2-(4-morpholino)-ethane sulfonic acid (MES), 0.5% glucose, 100 μ M acetosyringone (see **Note 1**).

2.4. β -Galactosidase Activity Assay

1. Spectrophotometer and tubes/cuvettes.
2. Vortex mixer.
3. Chloroform.
4. 0.1% Sodium dodecylsulfate (SDS).
5. Z buffer (per liter): 16.1 g $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$, 5.5 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.75 g KCl, 0.246 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.7 mL β -mercaptoethanol. Adjust pH to 7.0 using either H_3PO_4 or NaOH, as needed. Do not autoclave.
6. 30°C Water bath.
7. o-Nitrophenyl- β -D-galactoside (ONPG) solution: 4 mg/mL in Z buffer.
8. 1.0 M Na_2CO_3 .

3. Methods

3.1. *vir* Gene Induction

1. Grow *Agrobacterium* at 28–30°C (shake at 225–250 rpm) overnight in 5 mL YEP medium containing the appropriate antibiotics (see **Note 2**).
2. Dilute approximately 0.5 mL of the culture into 50 mL AB-sucrose minimal medium containing the appropriate antibiotics. Grow overnight at 30°C until the bacteria are in late log phase ($1\text{--}2 \times 10^9$ cells/mL. This represents Klett = 100 on a Klett-Somerson spectrophotometer, red filter; or $A_{600} = 0.8$; see **Note 3**).
3. Pellet the bacteria by centrifugation (5 min at $\sim 9000g$) in an SS-34 rotor, or 30 s in a microfuge).
4. Resuspend in 2 vol of induction medium containing 100 μ M acetosyringone. Shake very gently (approx 50 rpm, just enough to keep the bacteria suspended) 14–24 h at room temperature (not at 28–30°C).
5. Pellet the bacteria by centrifugation as in **step 3** above. Resuspend in MS plant tissue culture medium. Inoculate plants.

3.2. β -Galactosidase Activity Assay

1. After growth and induction of *A. tumefaciens* A348::mx358 (as described in **Subheading 3.1.**), centrifuge an aliquot (approximately 0.5 mL) of bacterial cells for 1 min in Eppendorf microfuge tubes. Resuspend the bacteria in a final volume of 4 mL Z buffer in the tubes used for the spectrophotometer.
2. Using Z buffer, adjust the concentration of cells such that the A_{600} is 0.1 to 0.25. Note this reading. Take out 2 mL of cells on which to perform the assays.
3. Add 2 drops of 0.1% SDS and 4 drops of chloroform to the 2-mL cell culture.
4. Vortex. Incubate the tubes in a 30°C water bath for 10 min.

5. Add 400 μ L ONPG solution, vortex, and start timing. Incubate the reaction at 30°C. Generally, reactions are run for 1–60 min. However, reactions can be run for several hours to detect low levels of β -galactosidase activity.
6. Terminate the reaction by the addition of 1 mL 1.0 M Na₂CO₃ (see **Note 4**).
7. Read the absorption at both 420 and 550 nm (see **Note 5**).
8. Calculate β -galactosidase activity as follows:

$$\text{units} = \frac{1000 \times (A_{420} - 1.75 \times A_{550})}{\text{time (min)} \times A_{600}}$$

Example:

$A_{600} = 0.15$, $A_{550} = 0.12$, $A_{420} = 0.63$ after a reaction time of 3.5 min.

$$\beta\text{-Galactosidase activity (in Miller units)} = \frac{1000 \times (0.63 - 1.75 [0.12])}{(3.5) \times 0.15} = 800 \text{ units}$$

4. Notes

1. Acetosyringone can be purchased from Aldrich Chemical Company (cat. no. D13,440-6). Keep the crystalline form of the chemical frozen in the dark at –20°C. The chemical can oxidize over time and will lose its potency. Immediately before use, make a 1000-fold concentrated stock solution in dimethylsulfoxide (DMSO). We do not freeze this stock solution but rather make it fresh each time.
2. It is crucial that bacteria be “fresh” and not taken from an older plate that has been sitting on the workbench for weeks. We routinely use bacteria freshly streaked from a stock (50% glycerol or 33% DMSO) frozen at –80°C.
3. The bacteria must be rapidly growing or they will not induce well. *Do not* grow the bacteria to greater than 10⁹ cells/mL and then dilute them. As the bacteria near the stationary phase of growth, they change their physiology and do not induce well.
4. Do not let the color turn a very deep yellow, because the reaction is no longer linear at this stage. The color will deepen when adding sodium carbonate.
5. During the assay, ONPG will be cleaved by β -galactosidase, yielding a yellow color that absorbs at 420 nm. The readings at 550 and 600 nm represent absorbance from cell debris or bacterial cells, respectively.

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II

MODEL PLANTS

Arabidopsis thaliana Floral Dip Transformation Method

Andrew Bent

Summary

Arabidopsis floral dip transformation is notable for a number of reasons. First, it is strikingly simple to perform. *Agrobacterium* is applied to flowering *Arabidopsis* plants that subsequently set seed, and transgenic plants are then selected among the progeny seedlings. Because no plant tissue culture is required, somaclonal variation is avoided, and the procedure can be performed easily by nonspecialists. Success rates are high: it is common that 1% of the progeny seedlings are transgenic. The biology behind the method is interesting: *Arabidopsis* and some related Brassicaceae are apparently unique in allowing exogenously applied *Agrobacterium* to colonize the interior of developing ovaries, where female gametophyte cell lineages are transformed. The availability of the method has had a transformative effect on the overall practice of plant molecular biology, as the generation and analysis of large numbers of transgenic plants is now routine in hundreds of laboratories. The method has been exploited in a genomics context to make stable gene knockout plant lines for most *Arabidopsis* genes.

Key Words: *In planta* transformation; germline transformation; *Arabidopsis thaliana*.

1. Introduction

The *Arabidopsis* “floral dip” method stands out from other plant transformation methods because it requires minimal labor and no plant tissue culture and can be performed successfully by nonspecialists. Flowering plants are simply dipped in or sprayed with an appropriate strain of *Agrobacterium tumefaciens*, and then the seed collected from these “T₀” plants is germinated under selection to identify transgenic “T₁” individuals. The method has been used in hundreds of laboratories throughout the world, where it is common for approximately 1% of T₁ seedlings to be transgenic. (Rates of 0.1–3% are typical.) The floral dip method (1) is a variant of the *in planta Arabidopsis* transformation method pioneered by Bechtold et al. (2), which offered major

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improvements to the equally pioneering work of Feldmann and Marks (3). For further background reading, see ref. 4.

Plant transformation has been possible for many years, but the floral dip method provides an instructive example of the qualitative shift in research progress that a simple, accessible transformation method can foster. Because transgenic plant lines can easily be generated for dozens of gene constructs, map-based gene cloning, structure/function studies of genes and proteins, signal transduction research, and studies of plant development, stress responses, and numerous other areas have all been significantly expedited. Investigators must wait 2–3 mo to obtain stably transformed plants while the seed matures on *Agrobacterium*-inoculated plants and selected T_1 seedlings are then grown, but this waiting time can be devoted to other research tasks. The most notable shift associated with the method may have been the arrival of gene knockout plant lines. High-throughput transformation capacity allows T-DNA to be used as an insertional mutagen (5). This was pushed a significant step further by sequencing the T-DNA insertion site for more than 340,000 transgenic *Arabidopsis* lines. Investigators can now obtain from stock centers a gene knockout line for most *Arabidopsis* genes (6; <http://signal.salk.edu/cgi-bin/tdnaexpress>).

In *Arabidopsis* floral dip transformation, essentially all T_1 transformants are the product of germline transformation events, meaning that the plants are uniformly transgenic in all cells. T_1 transformants are hemizygous at any given T-DNA insert locus because the T-DNA insertion events occur within the T_0 plant after divergence of the male and female gametophyte cell lineages (reviewed in ref. 4). The vast majority of transgenic plants arise from transformation of a female gametophyte (7–10). Therefore, every T_1 transgenic plant harvested off of the same T_0 individual carries a different T-DNA insertion. Because the targeted plant cells remain in their normal context within the plant and are not subjected to plant tissue culture before or after transformation, somaclonal variation is rare and has not been an issue for most users (11,12). Loci other than the site of a particular T-DNA insertion may undergo mutation, owing to another T-DNA insertion or to apparent T-DNA insertion/deletion events (13,14). However, the rate at which any particular gene is mutated remains exceedingly low, and unintended mutations usually become a factor only when large insertionally mutagenized populations are being used in forward genetic screens. In a study in which two different *Agrobacterium* strains carrying different T-DNA constructs were coinoculated onto *Arabidopsis*, roughly 15% of the transgenic plant lines carried both T-DNAs, and if both T-DNAs were present they almost always integrated together at the same insertion site (15). It is also intriguing that bacteria other than *Agrobacterium* have recently been used for floral dip transformation (16).

The floral dip transformation method has to date been successful only with *Arabidopsis thaliana* and some other Brassicaceae (17–21). Many concerted attempts have been made to use the method in other plant species, but these have failed. A report of success with *Medicago truncatula* (22) has unfortunately not been reproducible. Apparently, one reason that *Arabidopsis* is amenable to floral dip transformation is that the flower gynoeceium is open and accessible to exogenously applied *Agrobacterium* during early stages of floral development (8). A sufficient population of *Agrobacterium* can become established in the interior of the gynoeceium as ovules (the target of transformation) are beginning to develop. Subsequent closure of the gynoeceium prevents transformation in flowers that are at later stages of development. Successful transformation also requires that the host not manifest a strong necrotic reaction to the presence of *Agrobacterium*, which can cause abortion in flowers that have been colonized by *Agrobacterium*. Apparently, *Arabidopsis* and some other Brassicaceae satisfy the above constraints.

A concise, widely used version of the floral dip protocol for *Arabidopsis* is available at <http://www.plantpath.wisc.edu/fac/afb/protocol.html>.

2. Materials

2.1. Plant Materials

Any *Arabidopsis thaliana* may be used. *Arabidopsis thaliana* Col (“Columbia”) is the accession most commonly used by *Arabidopsis* researchers and is the accession for which the first *Arabidopsis* genome DNA sequence was determined (see **Note 1**). Ler (Landsberg *erecta*) and Ws (Wassilewskija) are also in widespread use. Most *Arabidopsis thaliana* accessions or ecotypes can be transformed by the floral dip method, often at rates similar to (within 10-fold of) those stated for Col-0 (see **Note 2**).

2.2. Agrobacterium Strain and Constructs

1. Many different *Agrobacterium tumefaciens* strain backgrounds have been used successfully in the floral dip method. GV3101 (23), more properly called GV3101 (pMP90), is the most commonly used strain, as it is particularly well suited for *Arabidopsis* transformation. Other strains of the C58 background carrying a disarmed C58 Ti plasmid can often be substituted with similar results. LBA4404 is also commonly used. LBA4404 generally works less well than GV3101, and this aspect of LBA4404 can be exploited to reduce the proportion of transformants that carry more than one T-DNA insertion.
2. A vast array of binary vectors has been used in floral dip transformation. The pCambia vectors are a particularly convenient and useful set of vectors for *Agrobacterium*-mediated plant transformation (http://www.cambia.org/pCAMBIA_vectors.html). Vectors that can be used with Gateway cloning

methods to make various transgene fusions are also available from a number of sources (e.g., 24; <http://www.psb.ugent.be/gateway/>)

2.3. Stock Solutions

1. Kanamycin (50 mg/mL): dissolve powder in dH₂O, filter-sterilize, and store frozen in 1-mL aliquots. Other antibiotics or herbicides may also be used as appropriate, for selection of *Agrobacterium* strains and plasmids, in *Agrobacterium* or in plants (see Notes 3–6).

2.4. Media

1. LB broth or LB agar (for growth of *Agrobacterium*): in 1 L deionized H₂O, add 10 g Bacto tryptone, 5 g yeast extract, 5 g NaCl. Add 15 g agar if making solid media. Autoclave prior to use. Add antibiotics only after media has cooled to temperatures below 65°C.
2. MS-agar (for selection of *Arabidopsis* transformants): 0.5X Murashige and Skoog macro- and micronutrients (example: Sigma, cat. no. M5524; use 2.15 g/L), 0.8% plant tissue culture agar (8 g/L). Autoclave, cool to 65°C, and then add 50 µg/mL kanamycin (or other appropriate selection agent; see Notes 4–6 regarding other plant selection media).

2.5. Other Solutions, Reagents and Supplies

1. 5% Sucrose: dissolve 50 g sucrose in 1 L of water. If made fresh in clean water, it is not necessary to autoclave or work under sterile conditions.
2. Silwet L-77 surfactant (GE Silicones/OSi Specialties, Wilton, CT). L-77 can be purchased in convenient quantities from Lehle Seeds (Round Rock, TX; <http://www.arabidopsis.com/>).
3. Peat mixture (for growing *Arabidopsis*): Sunshine Mix #1 (Sun-Gro Horticulture, Bellevue, WA) or Scott's MetroMix 360 (Hummert International, Earth City, MO).
4. Pots and plant flats (e.g., #F1020, no holes) can be purchased from Hummert International.

3. Methods

All plans for the generation of transgenic plants should be approved by proper institutional authorities. Regulated transgenic materials should be collected and rendered biologically inactive (e.g., autoclaved) prior to disposal.

3.1. Growing *Arabidopsis* for Transformation

To avoid unintended delays, it is necessary to plant *Arabidopsis* 4 to 8 wk in advance of the date on which you expect your *Agrobacterium* strains to be constructed and ready for plant inoculation.

There are many acceptable ways of growing *Arabidopsis* for floral dip transformation. The key is simply to grow healthy, vigorous plants that will pro-

duce plentiful seed. This is an important component of successful, reproducible floral dip transformation. If you have an *Arabidopsis* growth protocol that works well, use it. The following is one of many methods for *Arabidopsis* growth:

1. Grow *Arabidopsis* in a peat mixture such as Sunshine Mix #1 or Scott's MetroMix 360 (see **Note 7**). Moisten soil mix before adding it to pots. It should be moist enough to release water when squeezed, but with capacity to still take up more water.
2. Plant one or two seeds per 5-cm pot, or 9 to 20 seeds per 9-cm pot. After plants are established you will be bottom-watering, so place the pots in a flat that has no holes (such as a 1020 flat with a ridged bottom). Pack each pot with premoistened soil mix so that the mix is not loose enough to collapse with top watering but also not overly compressed. If you are planning to dip entire rosettes, grow plants through screen or mesh (see **Note 8**).
3. Plant *Arabidopsis* seeds by placing them on top of the moist soil mix. Do not bury the seed in the soil. For planting single seeds, pick them up by touching them with the fine tip of a moistened artist's 00 paintbrush. For planting a batch of a single genotype, suspend seeds (there are roughly 50 seeds per mg) in room temperature 0.1% agarose and dispense onto soil one at a time using a long-tipped Pasteur pipet. To plant larger flats with less precision, add a fixed amount of seed to a fine dry carrier such as dry crushed peat, and then disperse the mix evenly over the moist soil mix.
4. Cover the flats with a close-fitting clear plastic dome to maintain humidity. Many *Arabidopsis* accessions germinate more uniformly if held on wet soil at 4°C (dark is OK) for 2 to 5 d prior to being placed in the growth room. After flats are moved from 4°C to the growth room, leave the dome on at least until seedlings have emerged, cotyledons are green, and the first rosette leaf is clearly evident.
5. Then, over a few days, slightly displace (mis-seat) the domes to let in room air and reduce relative humidity gradually. After a few days of acclimation, remove domes entirely.
6. Grow plants at 22°C under long days (18+ h light/d) to encourage early bolting and flowering. *Arabidopsis* grows best at lower light levels than many plants (50–200 $\mu\text{E}/\text{m}^2/\text{s}$; 100–150 is optimal). Relative humidity of 50–70% is best, but lower humidity can be tolerated. Plants are likely to bolt (send up flowering stalks) 4 to 5 wk after germination, but this varies depending on your growth conditions. To grow larger plants that will generate more seed, see **Note 9**.
7. Pots should be watered from their base (add water to the flat; see **Note 10**).
8. Provide mild fertilization (e.g., quarter-strength MiracleGro) once every week or two. Many soil mixes contain a starter fertilizer that only lasts for the first week or two.
9. Keep insects away. Thrips especially, and aphids also, are serious pests on *Arabidopsis* that must be avoided whenever possible. Marathon is one insecticide that is useful if needed—add granules to soil and top-water it in. However,

the best approach with thrips or aphids is to purge the room of all plants, clean up the room, dry heat it for a few days, and then start over with new plants. Fungus gnats are OK at low population levels but can become a problem if not controlled. Fungus gnat numbers can be reduced by hanging yellow sticky paper in the growth area and by taking care to let the soil mix dry a bit between waterings. Spider mites tend to be more of a problem on older, neglected plants. To reduce insect problems, it is good to move older plants to a separate growth room for seed maturation and drying down.

No one likes to repeat experiments that fail for preventable reasons. Healthy plants and a green thumb are essential components to successful work!

3.2. Preparation of *Agrobacterium* Culture

Standard microbiological techniques can be used; see, for example, [ref. 25](#). A great deal of flexibility exists in the methods used to prepare *Agrobacterium* cultures for floral dip transformation of *Arabidopsis*. Cultures can be grown in LB, YEP, or other growth media. They can be grown to various stages of saturation and be resuspended at a variety of densities prior to plant inoculation (see [Note 11](#)). The following is one common and successful approach.

1. Prepare an *Agrobacterium tumefaciens* strain that carries the gene or genes of interest on a binary vector (see [Note 12](#)).
2. Use a single colony to inoculate and grow a small (1–5 mL) overnight culture in LB broth with antibiotic to select for the binary plasmid. If the strain has already been single-colony purified and you are in a hurry, a larger clump of cells can be used as starter inoculum (see [Note 13](#)).
3. The next day, dilute this starter culture approximately 1:100 into a larger volume of LB broth, with antibiotics added to select for the binary plasmid. For example, add 2.5 mL of starter to 250 mL in a 1-L flask and grow overnight at 28°C and 200 rpm.
4. The following day the culture should have grown at least to midlogarithmic phase and more likely will be approaching or at stationary phase (either is OK—grow longer if more cells are needed).
5. Transfer the liquid culture to a centrifuge bottle, and pellet cells by spinning for 10–20 min at room temperature at approximately 5500g.
6. Pour off supernatant into a flask or other waste container to which bleach can be added to kill stray *Agrobacterium* organisms.
7. Resuspend the pelleted cells in an equal volume of freshly made 5% sucrose solution.
8. Adjust by further dilution if necessary, to achieve $OD_{600} = 0.8$. (This is approximate: densities between 0.2 and 2.0 can work.) You will need 100–200 mL of *Agrobacterium* in sucrose for every two or three small pots to be dipped, or 400–500 mL for every two or three 3.5-inch (9-cm) pots. See [Note 11](#) regarding another possible shortcut.

7. Before dipping or spraying plants, add Silwet L-77 to a concentration of 0.05% (500 μ L L-77 per L of *Agrobacterium*) and mix well. If there are problems with L-77 toxicity, use 0.02% or as low as 0.005% L-77.

3.3. Plant Transformation

Transformation occurs within very young flowers. *Agrobacterium* is applied to floral buds by dipping the plant tissue in *Agrobacterium*, or by spraying the *Agrobacterium* onto the plant. Inoculated plants are kept at high humidity for the many ensuing hours and then grown to seed normally. Use of a surfactant enhances reproducibility by ensuring penetration of *Agrobacterium* into the flower interior.

1. Use plants that are bolting. Plants should carry many young unopened flower buds and only a few open or fertilized flowers. See **Note 14** regarding plant growth stages and **Note 15** for a discussion of plant numbers.
2. Dip plants into *Agrobacterium* solution prepared according to the **Subheading 3.2**. The *Agrobacterium* can be placed in a large beaker, disposable plastic soup bowl, or any other convenient container.
3. Swirl the plant tissues gently in the liquid for 1 or 2 s to remove bubbles and achieve full coverage, and then remove plant. It is only necessary to dip the inflorescence and not the rosette leaves, but you may wish to dip all above-ground plant tissues if some plants carry only very short inflorescences, or if the plants have been trimmed and shorter axillary inflorescences are being targeted. It is OK to reuse the *Agrobacterium* solution, but only for two or three different pots. See **Note 16** regarding spray-inoculation.
4. After dipping or spraying, it is preferable to maintain the plants at high relative humidity for a number of hours. This can be accomplished by placing plants under a clear plastic dome. If plants are too tall when inoculated they can be laid on their side in a clean flat and then covered with an inverted flat, some plastic wrap, or a clear plastic dome. The plants can then be left on a lab bench or returned to their normal growth room, but to avoid excessive heating, do not expose plants under a dome to direct sunlight.
5. The next day, remove the dome or other humidifying setup and return plants to their previous growing conditions.

3.4. Plant Care, Seed Harvest, and Storage

1. After plants have been inoculated with *Agrobacterium* and held at high humidity for 1 d, simply return the plants to their normal growing conditions and continue watering until the siliques start to lose their green color and become yellow. When most of the siliques on the plant are yellowing, it is OK to stop watering.
2. To reduce tangling and lodging, the bolts (flowering inflorescences) from a single pot can be tied together with string or twist-ties, with or without a small stake for support, or the bolts can be held by wrapping the pot in wax paper with the ends taped to form an open-topped tube. Plastic tubes for this purpose are commercially available (see **Note 17**).

3. Harvest seed once the siliques are notably brown and dry but before many siliques are spontaneously opening and dropping seed.
4. To harvest, hold the bolts from one pot over a clean piece of paper, and pull them gently between your fingers a few times to dislodge the seeds. Then pick up the paper and separate the siliques and stray stems from the seed, throw the siliques away, and pour the seeds into a 1.5-mL plastic tube or other container for storage. Use a new piece of paper for each seed lot. You can also cut the bolts off and place them unthreshed into an envelope, and then return at a convenient time to release seeds by gently crushing the envelope, tapping the heavy seeds into one corner, and then tearing off that corner of the envelope to transfer seeds into a container. Seeds do not have to be perfectly cleaned of plant debris, but they do need to be quite dry. Avoid harvesting moist seed by waiting for full maturity, or by only very gently agitating the bolts so that the moister seed remains adherent to the bolts (see **Note 17**).
5. Dry seeds can be stored at room temperature for many years with no dessication, but will keep better if stored at lower temperature and humidity. If some seeds are moist, store in paper envelopes in a sealed plastic box over dessicant, or punch a hole in the top of plastic tubes and store in a sealed box over dessicant.

3.5. Selecting Transformants

As with other steps, many different selection protocols may be used. The following protocol selects for kanamycin resistance. See **Note 5** for Basta and **Note 6** for hygromycin selection methods.

1. Prepare 15 × 150-mm Petri dishes containing approximately 65 mL of 50 mg/L kanamycin and 0.5X MS macro- and micronutrients (no sugar) in 0.8% plant tissue culture-tested agar.
2. Surface-sterilize 2000–6000 *Arabidopsis* seeds (40–120 mg):
 - a. Place seed in a 15-mL plastic centrifuge tube.
 - b. Add isopropanol, mix briefly, and then decant within 15 to 60 s.
 - c. Immediately add 50% bleach/50% water/0.05% Tween-20. Mix occasionally, and then, after 5 min, decant (see **Note 18**).
 - d. Rinse seed three to four times with sterile water, and decant.

See **Note 19** for an alternative, vapor-phase surface sterilization method.

3. To aid in seed dispersal across the selection plate, add sterile room temperature 0.1% agarose, using approximately 1.0 mL agarose for every 500 to 800 seeds.
4. Pour 3.5 to 4 mL resuspended seed onto each 150 × 15-mm selection plate. Gently tilt the plate and use a sterile curved glass rod spreader to guide the edge of the liquid evenly across the plate. Avoid the edge of the plate (the outmost 1 cm), since selection tends to fail in this zone. To avoid clumping of seed, do not move spreader around within the puddle of seeds on the selection plates—just use it to guide the edge of the puddle. Rinse spreader in 95% ethanol between seed batches to avoid cross-contamination.

5. Dry plates in sterile laminar flow hood until seeds no longer slide when plates are tipped. Cover plates. It is generally not necessary to wrap plates, but if this is desired, wrap with porous surgical tape.
6. Stratify seed on plates for 2 nights in refrigerator or cold room.
7. Move plates to growth chamber (50–100 $\mu\text{E}/\text{m}^2/\text{s}$, 18–24 h light, 21–24°C).
8. After 6 to 10 d, transformants should be clearly identifiable as dark green plants with healthy green secondary leaves and roots that extend well into the selective medium.
9. Using a pair of tweezers, gently remove plants from agar and transplant to previously moistened soil. Grow plants under a dome for roughly 1 wk, and then grow normally (see **Note 20**).
10. The phenotype of interest can often be scored directly on these primary transformants (the T_1 plants).

3.6. Zygosity, Progeny Testing, and Identification of Homozygous Transgenic Lines

After transgenic seedlings have been identified, the following points all merit consideration:

3.6.1. When to Start Using the Transgenic Plants

It is often not only possible but also desirable to do phenotypic tests on T_1 plants. Barring obvious problems (i.e., apparent 40 or 98% transformation success rate), the above selection protocols typically allow false positives (propagation of nontransgenic plants) at a rate below 1%. If your phenotype of interest is not affected by the selection and transplanting process, assay of a set of 10 to 20 T_1 plants has the added advantage that multiple independent transgenic lines will be tested, and a result will be obtained quickly. However, for important lines, the presence of the transgene and the phenotype must be confirmed in subsequent plant generations.

3.6.2. How to Identify True-Breeding Transgenic Lines

Because the T_1 plants are hemizygous, some T_2 progeny of self-fertilized T_1 plants will lack the transgene. Germination of T_2 plants under antibiotic/herbicide selection should remove nontransgenic plants and will also select for plants in which the selectable marker gene has not been silenced, allowing use in phenotypic testing, but this will not remove hemizygous plants from the population. Stable nonsegregating transgenic lines are often identified by pushing T_1 and T_2 generations through quickly and identifying the T_3 families in which no individuals lack expression of the selectable marker.

3.6.3. Are the Insertion Events Simple or Complex?

Many T_1 plants are likely to carry multiple insertion loci, and for this and other reasons these plants will not produce T_2 progeny families that exhibit

3:1 segregation for the transgene (see also **Subheading 2.2.**). Even if a single-locus T-DNA insert is present, the locus may carry a complex T-DNA structure with duplications, inverted repeats, T-DNA derived from transfer that extended beyond the right T-DNA border of the binary plasmid, and other things. Gene silencing is less likely to be an issue in lines that carry a single locus insert with one T-DNA copy at that locus, for reasons related to gene expression level as much as the absence of inverted repeat T-DNA inserts (**13,26**).

3.6.4. How to Identify Lines with Single-Copy Inserts

Isolate genomic DNA, cut with restriction enzyme (as described next), make a Southern blot, and probe the blot with a probe that matches insert DNA near the left T-DNA border. Choose a restriction enzyme and probe to detect restriction fragments that are generated by one cut within the T-DNA and by a second, unknown site outside the left T-DNA border in the host genomic DNA that flanks the insertion site. Note that this second site may be supplied by plant genomic DNA that flanks the T-DNA, or by adjacent T-DNA in a multimeric insert. If the transgenic line is likely to be of interest in many future experiments, it is often most efficient to identify simple insertion lines using T_1 leaf tissue, allowing disposal of less desirable T_1 lines prior to seed harvest and further expenditure of effort. Alternatively, one can use genomic DNA pooled from multiple T_2 individuals within a family and test only those T_2 families that exhibited 3:1 segregation for the selectable marker or the gene of interest.

3.6.5. How Many Different Transgenic Lines to Save for a Given Construct

There is no single correct answer to this; the answer depends in part on the importance of that particular transgene in your research and on the effort spent to identify simple insertion events. Owing to variations in transgene expression caused by site of chromosomal insertion and owing to the possibility of secondary unintentional mutations in the genome of any single transgenic line, at least three independent lines with a simple insert structure should be saved. If work has not been done to identify lines that carry a simple insertion, the likelihood of future gene silencing makes it wise to save a few dozen independent transgenic lines.

4. Notes

1. Col accessions with different numbers (e.g., “Col-4”) are progeny from the original Col-0 line, and are for the most part identical to each other. *Arabidopsis* seed stocks and other resources can be obtained from the *Arabidopsis* Biological Resource Center (at Ohio State University, Columbus, OH; <http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm>) or from the European

Arabidopsis Stock Center (Nottingham, UK; <http://arabidopsis.org.uk/>). Numerous other *Arabidopsis* resources can be accessed online through The *Arabidopsis* Information Resource (<http://www.arabidopsis.org/index.jsp>), which is a highly recommended site.

2. Most *Arabidopsis* ecotypes (wild-type accessions) have been transformable by this method. Some accessions are more difficult to transform than others, but as long as the transformation rate is above roughly 1 in 10,000 T₁ seedlings, the stated protocols can be carried out with minimal added burden. Reports of transformation recalcitrance can vary from laboratory to laboratory, and the causal variables have generally not been identified. If working with novel accessions or with mutations that may impact transformation rate, include wild-type Col-0 as a control to establish a baseline transformation rate that is specific to your experiment. If a recessive mutation is responsible for poor transformation, transform a heterozygote and then identify progeny with the desired genotype.
3. For selection of *Agrobacterium tumefaciens* carrying an appropriate antibiotic resistance gene, the following antibiotics can be used with only normal care:
 - a. Kanamycin: 25–100 µg/mL.
 - b. Rifampicin: 50–100 µg/mL (Rifampicin stocks can be made at 20 mg/mL in methanol. They are light sensitive and should be stored in the dark.)
 - c. Gentamicin: 50–100 µg/mL.
 - d. Erythromycin: 150–200 µg/mL.
 - e. Carbenicillin: 100 µg/mL.
 - f. Spectinomycin: 100 µg/mL.

Other antibiotics require more caution:

- g. Tetracycline: 2–2.5 µg/mL. If possible, avoid this antibiotic with *Agrobacterium*. Higher levels of tetracycline will kill strains expressing only the plasmid-derived resistance and with C58-derived strains such as GV3103 will select for the spontaneous tetracycline resistant isolates that do not carry the binary plasmid (27). Mutations to tetracycline resistance arise spontaneously at high frequency (10⁻⁶–10⁻⁵). Because this spontaneous resistance is typically more effective than the resistance expressed from binary plasmids, the smaller tetracycline-resistant colonies are often the ones to pick—they are more likely to contain the strain + plasmid that you probably seek, whereas the larger colonies are spontaneous mutants. Note also: if your binary plasmid encodes tetracycline resistance but also contains a 35S-*nptII* construct (for kanamycin resistance in plants), the latter is often expressed effectively in *Agrobacterium* (but not in *E. coli*). So, with this type of plasmid, you may be able to select for tetracycline resistance in *E. coli* (10 µg/mL) and then kanamycin resistance (50 µg/mL) in *Agrobacterium*.
- h. Chloramphenicol: it is generally a good idea to avoid use of chloramphenicol entirely with *Agrobacterium*.
- i. Streptomycin: use with some care, at 100–200 µg/mL. Some strains give rise to spontaneous streptomycin-resistant mutants at relatively high frequency.

If in doubt about the presence of your binary plasmid in *Agrobacterium*, this can be checked by polymerase chain reaction (using appropriate positive and negative controls), or by Southern blots, or by successful rescue of the plasmid back into *E. coli* (see Chapter 5).

4. For kanamycin selection and other plate-based selections, plant tissue culture vitamins are not needed for the brief period that *Arabidopsis* will grow on these plates. However, if present, the vitamins can be autoclaved (e.g., Sigma, cat. no. M6899).
5. Basta selection: with *Arabidopsis* floral dip, a common alternative to kanamycin selection is Basta selection. Basta is a commonly used trade name for phosphinothricin, which is also called glufosinate. One advantage of Basta selections is that they can be carried out in a greenhouse using plants grown in soil. To identify *Arabidopsis* plants expressing a Basta resistance gene, Liberty herbicide (AgrEvo USA Company, active ingredient glufosinate, 200 mg/L) can be diluted 1:1000 (1 mL/L) and then sprayed on germinated *Arabidopsis* seedlings once every 3 d for approximately 2 wk after seedlings germinate and emerge. Basta selections can also be done on 0.5X MS/0.8% agar plates.
6. Hygromycin selection: although kanamycin or Basta is preferable for *Arabidopsis*, hygromycin selection is another option. However, past hygromycin selection protocols for *Arabidopsis* have been variable and problematic for many researchers. The following greatly improved protocol was supplied by Dr. Zhiyong Wang (Carnegie Institute of Washington, Stanford, CA):
 - a. Pour 0.5X MS/0.8% agar plates containing 25 mg/L hygromycin.
 - b. Sow surface-sterilized *Arabidopsis* seeds (up to 4000 seeds per 150-mm plate).
 - c. Stratify in cold for 2 to 3 d.
 - d. Put plates at room temperature in light for 4–12 h to promote germination.
 - e. Move plates into the dark at room temperature (wrap in foil, and put in a cupboard). Grow in the dark for 5 d after the brief light treatment.
 - f. After 5 d, hygromycin-sensitive seedlings will be lying on the growth medium and have short hypocotyls and open cotyledons. Hygromycin-resistant seedlings will be standing up, with elongated hypocotyls and closed cotyledons. Mark the resistant seedlings and then transfer the plate to the lab benchtop for 2 d to allow seedlings to green up and recover in gentle light.
 - g. Transfer hygromycin-resistant seedlings to moist soil and harden off gradually over a few weeks. (Grow under a dome with high humidity initially.)
7. Sunshine Mix #1 or Scott's MetroMix 360 are good potting mixes to use. Other mixes can work, but note that many mixes do not work well for *Arabidopsis*.
8. For some experiments it is desirable to immerse entire low-lying rosettes into an *Agrobacterium* suspension, to achieve inoculation of shorter young inflorescences. In this case you may want to grow plants through screen or mesh (tulle, bridal veil, cheesecloth) that holds the soil in when you immerse the plants in liquid. When initially planting, mound the moistened soil in a smooth, relatively flat dome that extends roughly 1 cm above the top rim of the container, and then

cover with mesh held around the edges by a strong rubber band so that the mesh stays very close to the soil surface. Otherwise, young germinating seedlings will expand their cotyledons prior to penetrating the mesh and will get stuck underneath the mesh.

9. To delay bolting and to encourage sustained growth of larger plants that will produce more seed when they bolt, grow under short days (8–10 h light/d, with no light allowed to disrupt the 14+ h dark periods) and then shift to longer days (18+ h light/d). Allow a transition period to avoid plant stress caused by the shift to more light, for example, by using shade cloth for the first few days after the shift, or, if using a controlled environment chamber, simply interrupt the dark period with a 15-min light interval once every 4 h so that the plants initiate bolting.
10. Do not overwater; soil should become thoroughly wetted, but excess water should be gone from the tray within a day, and soil should start to dry slightly prior to the next watering. Excessive watering will encourage fungus gnats.
11. U.S. patent no. 6,353,155 B1 (Klöti, A. S. and Mulpuri, R., 2002. “Method for transforming plants”) describes floral dip transformation using liquid LB cultures of *Agrobacterium* that are diluted approximately three- or fourfold in 5% sucrose/0.05% L-77, with no centrifugation to remove the LB. This is particularly useful if many *Agrobacterium* strains will be used.
12. *Agrobacterium* is typically grown at 28 to 30°C (growth at temperature closer to 37°C can cause Ti plasmid loss or lethality). Be sure to work with a single colony-purified isolate. Strains may be stored temporarily at 4°C for 1 to 3 wk as a Petri dish culture (wrapped to maintain humidity). If you will be saving and reusing a strain, make a frozen permanent stock that is stored in 14% glycerol or 7% dimethyl sulfoxide at –70°C. (Example: add 0.33 mL sterile 40% glycerol to 0.67 mL of a recently saturated overnight culture grown in liquid LB, mix well, and place in freezer.) Do not serially propagate a strain from plate to plate over many passages. Work with Petri dish cultures that are started fresh every few weeks from your frozen permanent stock.
13. *Agrobacterium* grows more slowly than *E. coli*, usually taking more than overnight to form single colonies of significant size on a plate. Use of multiple antibiotics may slow the growth of *Agrobacterium* further, even though the *Agrobacterium* is resistant to those antibiotics. If you want liquid cultures to be ready the next day, use a bit more starter inoculum than you might with *E. coli* and make sure that the cultures are well aerated. It is also possible to skip the overnight starter culture and inoculate a larger volume of liquid media with cells from a plate or from a freezer stock, although the rate of culture growth may be quite unpredictable. If only a few plants will be inoculated, a fresh lawn of bacteria from a Petri dish can be resuspended in liquid to provide sufficient inoculum.
14. Keep in mind that the targets for inoculation are immature flower buds that will open (reach anthesis) 4 to 10 d after the date of inoculation. These buds should directly contact the *Agrobacterium* solution. Although there is an “optimal” date for inoculation, you will probably have a period of 10+ d across which inoculation can successfully produce transformants. Inoculate the plants only once, or if

percent efficiency is important, no more than twice, with a 7-d interval between inoculations. To delay use of plants that are bolting before your *Agrobacterium* strains are ready, young inflorescences can be clipped off to promote growth of secondary inflorescences, providing an extra 5 to 7 d with each clipping. This can be done a few times if needed. If you are clipping, be sure the plants remain well fertilized and healthy.

15. How many plants do you inoculate to get 10 transformants? If you assume a transformation rate of only 0.1%, then you will need 10,000 seeds to get 10 transformants. You can expect to harvest a few thousand seeds off of every *Arabidopsis* plant if the plants are well spaced and healthy. Plants grown at high density and plants that flower rapidly (for example, owing to growth under 24 h/d lighting) may produce only 500–1000 seeds. In our laboratory, a single pot of nine *Arabidopsis* plants is often dipped for each binary plasmid construct, with all the seed from that pot harvested together into a single tube. Beginners may want to dip three pots for each construct.
16. For high-throughput transformation projects in which many plants will be inoculated with only one or a few *Agrobacterium* strains, spraying may be preferable to dipping. Prepare bacteria + surfactant as described for dip inoculation, but place in a small pressure sprayer or atomizer and spray this directly onto inflorescences, targeting clusters of young unopened flower buds. An advantage of spraying is that fewer bacteria need to be grown; however, the sprayer can be less convenient than simple dipping. Note that cross-contamination is possible if you are spraying adjacent plants with more than one *Agrobacterium* strain. Note also that Silwet L-77 can injure corneal tissues—this is especially an issue if you wear contact lenses.
17. Cross-fertilization (unintended outcrossing) of adjacent *Arabidopsis* plants is very rare (1:10,000 or less). However, cross-contamination of seed lots is likely if plants that were inoculated with different *Agrobacterium* strains become tangled during subsequent growth, or if seed harvest is not carried out in a tidy fashion with care and constant attention to avoid stray seeds. Contamination is quite easy to accomplish; discovering and correcting a contamination event can be very costly.
18. During seed surface-sterilization, it is convenient to remove liquids using a disposable yellow pipet tip attached to a continuous suction device (such as a Pasteur pipet-rubber tubing-liquid trap-house vacuum device). Use a new tip for each tube, and use additional care to avoid accidental transfer of seed between tubes. A brief centrifugation in a clinical centrifuge may help to minimize seed loss during decanting if necessary. Sterilize only the seed that you will plate. It is advisable to include, as a positive control for selection, sterilized seed from a known transformed variety. (Place a few positive control seeds onto a marked location on a few of the selection plates.) Sterile seed should not be kept in the 0.1% agarose solution for more than 24 h.
19. Vapor-phase surface sterilization of *Arabidopsis* seed: the following protocol contains a convenient method for surface sterilization of plant seeds. In particu-

lar, the method does not require all the soaking and rinsing of liquid-phase bleach-sterilization methods. Although this protocol substantially reduces hands-on manipulations, it does require some incubation time (a few hours to overnight). This vapor-phase sterilization protocol was adapted from a version supplied by Maud Hinchee and colleagues at Monsanto.

- a. Obtain a vessel for seed sterilization, typically a dessicator jar. Place in fume hood.
- b. Place seed that is to be sterilized into appropriate resealable containers (for example, microcentrifuge tubes).
- c. Place open containers of seed into a rack or stand inside the dessicator jar.
- d. Place a 250-mL beaker containing 100 mL bleach into the dessicator jar.
- e. Immediately prior to sealing the jar, carefully add 3 mL concentrated HCl to the bleach.
- f. Seal jar and allow sterilization by chlorine fumes to proceed for a period of between 3 and 16 h. The time needed will vary based on the configuration of seed and the extent to which seed is contaminated. Three to four hours is often sufficient for reasonably clean seed. Overnight is usually acceptable, although some seed killing may occur, especially if seed is not fully mature and dry.
- g. Depending on the application, open container in fume hood or in sterile laminar flow hood, seal microfuge tubes or other seed containers, and remove surface-sterilized seed for use.

Caution: chlorine gas is poisonous to humans—work with proper ventilation.

20. Transplanting success is improved by using tweezers to break up the agar around the root prior to pulling on the plantlet, by using your fingers to gently remove any adhering chunks of agar from the root before planting, by using a squirt bottle to saturate the soil with water immediately after transplanting (keep the rosette leaves up above the soil), and by growing plants under a dome (for high humidity) for the first several days. Larger (older) plants are often more robust for transplanting, but larger root systems can be more difficult to remove from the agar. If you break up the roots severely, put the plantlet onto a new selection plate or a sucrose/MS plate for a few days to allow new root growth before transplanting.

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***Agrobacterium* Transformation of *Arabidopsis thaliana* Roots**

A Quantitative Assay

Stanton B. Gelvin

Summary

Arabidopsis thaliana has become a major model system for investigating plant molecular, genetic, and biochemical processes. *Arabidopsis* is highly susceptible to *Agrobacterium*-mediated transformation using “flower dip” and “vacuum infiltration” protocols. However, *Arabidopsis* has also become a major system to investigate the mechanism of *Agrobacterium*-mediated transformation of somatic tissue. Such investigations require a reproducible, quantitative assay system to determine transformation frequency. We describe here an *Arabidopsis* root transformation protocol that can be used to determine transformation frequencies for wild-type and mutant *Agrobacterium* strains and for various *Arabidopsis* wild-type and mutant lines.

Key Words: *Agrobacterium tumefaciens*; *Arabidopsis thaliana*; plant genetic transformation; plant genetic engineering; root transformation.

1. Introduction

Agrobacterium-mediated genetic transformation has become an essential aspect of many investigations of plant molecular, genetic, physiological, biochemical, cell, and developmental processes. *Agrobacterium* can transform somatic tissues of a large number of higher plant species (1–5). In addition, *Agrobacterium* can transform female gametophytic tissues of a few plant species (6–17). However, there are only a few transformation assays described in the literature that are even semiquantitative (18,19). It is often important for many studies to compare the virulence of numerous *Agrobacterium* strains or bacterial mutants. In addition, recent work has focused attention on the role of host plant genes in the transformation process (20–27). We have therefore

devised a quantitative *Arabidopsis* root transformation assay that allows one to determine the relative transformation efficiency of different *Agrobacterium* strains or of different *Arabidopsis* ecotypes and mutants.

The root transformation assay described here can easily be adapted to test the virulence of numerous *Agrobacterium* strains or the transformation competence of numerous *Arabidopsis* mutants/ecotypes in parallel. We suggest that the investigator initially perform stable tumorigenesis assays, using *A. tumefaciens* A208 or a similar nopaline-type strain. This strain generally is more virulent on *Arabidopsis* roots than are other tumorigenic strains, such as the octopine-type strain A348 or the “super-virulent” succinamopine-type strain A281 (22). In addition, *A. tumefaciens* A208 incites large, green teratomas on the roots of many *Arabidopsis* ecotypes (Fig. 1A). It is therefore easier to distinguish a highly sensitive plant response from a weaker response, which generates small, green or yellow unorganized tumors. However, some ecotypes such as Col-0 generally respond to this *Agrobacterium* strain by generating smaller, yellower unorganized tumors rather than large green, leafy teratomas (25).

Because crown gall tumors result from a response of the plant to the increased levels of phytohormones directed by *Agrobacterium*, plant “hormone response” mutants may appear to be “rat” (resistant to *Agrobacterium* transformation) mutants (23). However, these plants may actually be transformed (i.e., T-DNA transferred to and integrated into the plant genome); they just lack the appropriate response to increased hormone levels. We therefore suggest that the investigator employ other transformation assays. These include transformation to antibiotic (e.g., kanamycin or hygromycin) or herbicide (e.g., phosphinothricin) resistance (25). In addition, one can perform transient transformation assays and screen for generation of β -glucuronidase (GUS) activity directed by a *gusA*-intron gene (25,28; Fig. 1B). Generation of such transient GUS activity requires transfer and nuclear translocation of the T-DNA, as well as conversion of the single-stranded T-strand to a double-stranded transcription-competent form. However, transient expression of the *gusA*-intron gene does not require integration of the T-DNA into the plant genome (29).

The following protocol has been developed and used in our laboratory for approximately 10 yr. Although the assay is labor intensive, it is quantitative and has served us well to investigate both the virulence of various *Agrobacterium* strains and the transformation competence of various *Arabidopsis* ecotypes and mutants. Skilled researchers can process 50–70 plants per day. As with all techniques, attention to detail is important. Our experience has been that people who attempt to “cut corners” generally get more variable results than those who follow the protocol carefully.

The protocol described below is not meant for generating transgenic *Arabidopsis* plants using a root transformation protocol. Others have described such methods (30).

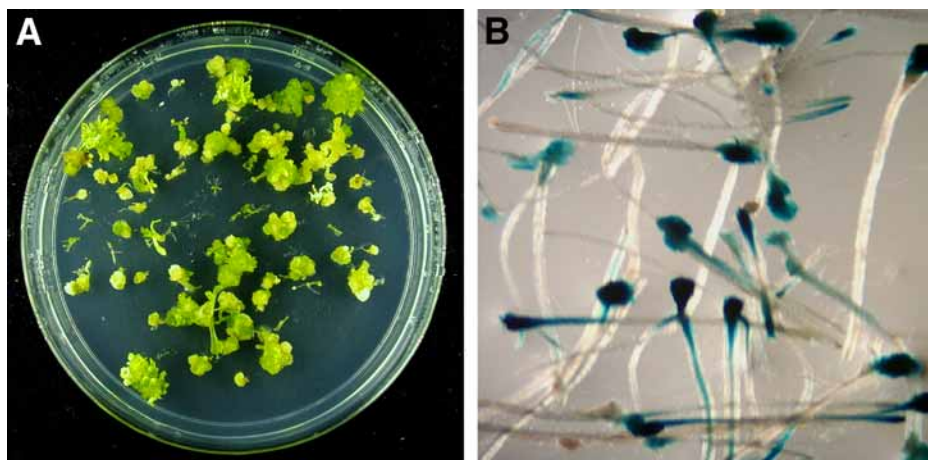


Fig. 1. **(A)** Crown gall tumors developing on root segments of *Arabidopsis thaliana* ecotype Ws. Root segments were inoculated with *A. tumefaciens* A208 and photographed approximately 1 mo later. Note the large green teratomas generated with this combination of *Agrobacterium* strain and *Arabidopsis* ecotype. **(B)** GUS activity in the infected areas of *Arabidopsis* root segments. Root segments were inoculated with a nontumorigenic *Agrobacterium* strain containing a T-DNA binary vector with a *gusA*-intron gene. The segments were stained with X-gluc 6 d after infection.

2. Materials

2.1. Plant Material

1. *Arabidopsis thaliana* seed. Several ecotypes are easily transformed by *Agrobacterium*, including Columbia and Ws (31). Seeds can be obtained from the Ohio State University *Arabidopsis* Biological Resource Center (<http://arabidopsis.org/servlets/Order?state=catalog>).

2.2. Agrobacterium Strains

These strains can be obtained from the author.

1. *A. tumefaciens* A208 (for tumorigenesis assays).
2. *A. tumefaciens* At849 (for kanamycin-resistance and GUS assays).
3. *A. tumefaciens* At872 (for phosphinothricin-resistance assays).

2.3. Growth and Preparation of Agrobacterium

1. Sterile 0.9% NaCl.
2. YEP medium (per liter): 10 g peptone, 10 g yeast extract, 5 g NaCl. If the medium is to be used in Petri plates, add 15 g Bacto agar before autoclaving. It is not necessary to adjust the pH.
3. Spectrophotometer.

2.4. Growth of Arabidopsis and Preparation of Root Segments

1. MS basal medium (for 1 L): 4.32 g MS minimal salts (Gibco), 0.5 g 2-(4-morpholino)-ethane sulfonic acid (MES), 1 mL vitamin stock solution (1000X), 10 mL myoinositol stock solution (100X), 10 g sucrose. Adjust pH to 5.7 with 1 N KOH; add 7.5 g Bacto agar. Autoclave for 20–30 min.
2. Callus Inducing Medium (CIM; for 1 L): 4.32 g MS minimal salts (Gibco), 0.05 M MES, 1 mL vitamin stock solution (1000X), 10 mL myo-inositol stock solution (100X), 20 g glucose, 1 mL indole-3-acetic acid (IAA) stock solution (1000X), 0.5 mL 2,4-dichlorophenoxy acetic acid (2,4-D) stock solution (2000X), 0.5 mL Kinetin stock solution (2000X). Adjust pH to 5.7 with 1 N KOH; add 7.5 g Bacto agar. Autoclave for no more than 20 min.
3. B5 Medium: Gamborg's B5 medium (Gibco; basal medium with minimum organics). Dissolve the entire contents from one bottle to make 1 L medium. If the medium does not come with sucrose as a component, add 20 g sucrose. Adjust pH to 5.7 with 1 N KOH; add 7.5 g Bacto agar.
4. Stock solutions:
 - a. Myoinositol (100X): 10 mg/mL.
 - b. Vitamins (1000X): 0.5 mg/mL nicotinic acid, 0.5 mg/mL pyridoxine, 0.5 mg/mL thiamine-HCl.
 - c. IAA (1000X): 5 mg/mL in H₂O. (Add a small amount of KOH to help dissolve.)
 - d. 2,4-D (2000X): 1 mg/mL in H₂O. (Add a small amount of KOH to help dissolve.)
 - e. Kinetin (2000X): 0.6 mg/mL H₂O. (Add a small amount of KOH to help dissolve.)
5. Sterile H₂O.
6. Sterile filter paper.
7. Forceps, scalpels, burners, sterile tissue culture hood, and other standard materials for performing plant tissue culture experiments.
8. Petri dishes and baby food jars with snap-on plastic lids. Magenta boxes can be used instead of baby food jars, but they are generally larger than what is needed and will therefore waste growth chamber space.
9. Parafilm.
10. Plant growth chamber.
11. Timentin.
12. Bleach.
13. Sodium dodecyl sulfate (SDS).

2.5. Arabidopsis Transformation Assays

1. Antibiotics/herbicides for plant selection (final concentrations in mg/L): 50 kanamycin; 20 hygromycin; 10 phosphinothricin.
2. X-gluc staining solution: 50 mM sodium phosphate, pH 7.0, 0.1% Triton X-100, 1–2 mM X-gluc.

3. Methods

3.1. Seed Sterilization

1. Sterilize *Arabidopsis* seeds for 10 min in a solution of 50% bleach plus 0.1% SDS.
2. Rinse five times with sterile distilled H₂O.
3. Using a P200 pipeting device, place 50 seeds onto a Petri plate containing B5 medium (either lacking selective antibiotics or containing 50 mg/L kanamycin, 10 mg/L phosphinothricin, or 20 mg/L hygromycin B, whichever is appropriate). Also put into the medium 100 mg/L Timentin to inhibit growth of any bacteria that may be trapped under the seed coat.
4. Place plates at 4°C for 2 d.

3.2. Plant Growth

1. Germinate seeds in a growth chamber (23°C, 14-h light, 10-h dark) for 7 to 10 d, until the plants have at least one pair of “true leaves.”
2. Transfer seedlings into a baby food jar containing B5 medium without antibiotics and grow for at least 10 d. Plants are ready for processing when the roots are long enough to get a minimum of 60 segments (roots are 1–2 segments long). Plants should be processed before a flower bolt emerges (see **Note 1**).

3.3. Preparation of Agrobacteria for Transformation

1. Grow *A. tumefaciens* at 30°C on a YEP plate containing the appropriate antibiotics to select for the presence of any T-DNA binary vector plasmid (see **Note 2**).
2. Inoculate *Agrobacterium* into 5 mL YEP medium containing the appropriate antibiotics and grow overnight at 30°C with shaking. The next day, dilute the bacteria 1:20 into 50 mL YEP medium containing the appropriate antibiotics and grow at 30°C with shaking until the bacteria reach a density of 10⁹ cells/mL (Klett = 100; or A₆₀₀ = 0.8; see **Note 3**).
3. Wash the cells once in 0.9% NaCl, and then resuspend the pellet in 0.9% NaCl at a concentration of 10⁸ cells/mL (see **Note 4**).

3.4. Preparation and Inoculation of Arabidopsis Root Segments

1. Gently remove the *Arabidopsis* plants from the baby food jars, dip the roots into sterile H₂O, and lay the plants down in a Petri dish. Pour a small amount of sterile H₂O into the dish and “align” the roots together.
2. Cut the roots from the base of the plant and replace the shoots back into B5 medium in baby food jars. Give each plant a code number. Cut the roots into 0.3- to 0.5-cm-long segments.
3. Transfer bundles of root segments onto MS basal medium without antibiotics in a Petri dish (see **Note 5**). Place 2–3 drops of bacterial solution onto each bundle to cover the root bundles and leave them for 10 min (see **Note 6**).
4. Remove most of the bacterial solution with a Pipetman, seal the Petri dishes with parafilm, and coculture the bacteria and root bundles for 40 to 50 h in a growth chamber at 20°C. Incubation can be either in the light or in the dark.

5. At 40 to 50 h after cocultivation, rinse the segments with sterile distilled H₂O containing Timentin (100 mg/L) or scrape off infected root segments on the surface of the medium (see **Note 7**).
6. Transfer roots onto different types of medium according to the assay you are going to perform (see **Subheadings 3.5.** and **3.6.**). For primary screening for *rat* mutants, separate roots into small bundles (up to five root segments/bundle). For secondary screening and quantitation, separate into individual root segments; do not use root bundles. You should have a minimum of 60 root segments per plate; each plate represents an individual plant.

3.5. Transient GUS Transformation Assay

1. For transient GUS assay, transfer root bundles onto Callus Inducing Medium (CIM) containing 100 mg/L of Timentin, seal the plates with double layers of parafilm, and leave them at 23°C.
2. After an additional 3 to 6 d, take out the root segments and stain them in X-gluc solution overnight at 37°C.

3.6. Stable Transformation Assays

3.6.1. Score Crown Gall Tumorigenesis

1. If one uses the tumorigenic strain *A. tumefaciens* A208 and wishes to score for tumorigenesis, transfer the roots onto MS basal medium with 100 mg/L Timentin (to kill *Agrobacterium*).
2. Seal the plates with double layers of parafilm and place them in a growth chamber at 23°C for 4 to 5 wk. Approximately 2 wk after infection, you should be able to see small tumors appearing.
3. When scoring for the phenotype of tumorigenesis, not only must you record the percentage of root segments that give tumors, you must also indicate the morphology of the tumor (small yellow, large yellow, small green, or large green). The percentage of each morphology class should also be recorded. (You may wish to use a bar graph in which the bar is divided into four sections of different colors, each color representing a morphology class.)

3.6.2. Score Antibiotic- or Herbicide-Resistant Transformants

1. If one uses an *Agrobacterium* strain carrying a binary vector, one can score transformation by scoring antibiotic- or herbicide-resistant calli. Transfer the roots onto Callus Inducing Medium (CIM) containing 100 mg/L of Timentin and either 50 mg/L of kanamycin, 20 mg/L of hygromycin, or 10 mg/L of phosphinothricin (whichever is appropriate).
2. Seal the plates with double layers of parafilm and place them in a growth chamber for 4 to 5 wk. Approximately 2 wk after infection, you should be able to see small yellow calli appear.

4. Notes

1. It is important that the plants be healthy and the roots grow well and quickly. Roots that do not grow well generally do not transform well.
2. It is crucial that bacteria be “fresh” and not taken from an older plate that has been sitting on the workbench for weeks. We routinely use bacteria freshly streaked from a stock (50% glycerol or 33% DMSO) frozen at -80°C .
3. The bacteria must be rapidly growing or they will not transform well. *Do not* grow the bacteria to greater than 10^9 cells/mL and then dilute them. As the bacteria near the stationary phase of growth, they change their physiology and do not transform well.
4. Centrifugation of bacteria is done in a microfuge at top speed for 1 min. For certain experiments, you may wish to resuspend the bacteria at 10^7 cells/mL, or even less.
5. You should be able to place approximately 20 “bundles” of root segments (from 20 plants) into a single dish.
6. *All root segments must be infected within 30 min of being cut.* Do not leave the segments for longer periods before infection. The root segments will not transform well if left for longer periods.
7. Do not leave the roots to coculture for more than 50 h. *Agrobacterium* will overgrow the root segments and may kill the plant tissue.

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Medicago truncatula* Transformation Using Leaf Explants

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Summary

Legumes have long been recalcitrant to efficient *Agrobacterium tumefaciens*-mediated transformation. The choice and use of model legume plants (*Medicago truncatula* and *Lotus japonicus*) for molecular studies has triggered extensive studies devoted to the development of efficient *Agrobacterium*-mediated transformation protocols for these two plants. In *M. truncatula*, transformation protocols rely on the use of highly regenerable lines obtained by recurrent in vitro culture selection. These protocols are based on *Agrobacterium*-mediated transformation of *M. truncatula* followed by somatic embryogenesis-mediated plant regeneration. We describe here the protocol developed for *M. truncatula* R108-1 (c3).

Key Words: *Medicago truncatula*; legumes; transgenic plants; in vitro culture.

1. Introduction

Several *Agrobacterium*-based transformation protocols have been developed for *Medicago truncatula* in the last 10 yr (**1–8**). These protocols rely on the use of lines selected by recurrent in vitro culture selection and regeneration ability. Here we describe a procedure developed for the highly embryogenic *M. truncatula* line R108-1 (c3) (**5**, www.isv.cnrs-gif.fr/embo01/). Other protocols were developed for the embryogenic A17 Jemalong genotype 2HA3-9-10-3 (named 2HA; **1,2,7**). In each case, the developed protocol works for one line but normally not for the others, suggesting that these lines represent mutant lines that have acquired an improved regeneration capability when following this special

*This work is dedicated to Dr. Hanh Trinh who initiated this protocol.

Table 1
Composition of the N6 Major Salts^a

Chemicals	Amount for 1 L	Final concentration in the stock solution (mM)
MgSO ₄ ·7H ₂ O (dissolve completely)	1.85 g	7.5
KNO ₃	28.30 g	280
(NH ₄) ₂ SO ₄	4.63 g	35
CaCl ₂ ·2H ₂ O	1.66 g	11
KH ₂ PO ₄	4.00 g	30
H ₂ O	QSP 1 L	—

^aSee **ref. 11**. This solution can be stored at 4°C.

Table 2
Composition of the SH Minor Salt^a

Chemicals	Amount for 100 mL	Final concentration in the stock solution (mM)
MnSO ₄ ·H ₂ O	1 g	60
H ₃ BO ₃	500 mg	80
ZnSO ₄ ·7 H ₂ O	100 mg	3.5
KI	100 mg	6
Na ₂ MoO ₄ ·2 H ₂ O	10 mg	1
CuSO ₄ ·5 H ₂ O	20 mg (CuSO ₄ = 12.8 mg)	0.8
CoCl ₂ ·6 H ₂ O	10 mg	0.4
H ₂ O	QSP 100 mL	—

^aSee **ref. 12**. This solution can be stored at 4°C without autoclaving.

protocol. They are generally based on a callus-inducing phase followed by somatic embryogenesis and plant regeneration.

The regeneration protocol developed in our laboratory for R108-1 (c3) routinely allows 80% of the explants to form calli that give rise to numerous embryos and plantlets. When transformation is performed, between 50 and 80% (depending on the selectable marker used) of the original explants will give transgenic embryogenic calli that themselves can produce dozens of transgenic plants. Thus, our transformation efficiency can be as high as 50 transgenic plants (Southern positive) from 100 infected leaf explants. This very high transformation/regeneration efficiency has allowed planning experiments requiring several hundreds of plants (**9,10**).

Table 3
Composition of the SH Vitamin Solution^a

Chemicals	Amount for 100 mL	Final concentration in the stock solution (mM)
Nicotinic acid	500 mg	40
Thiamine HCl (vitamin B ₁)	500 mg	15
Pyridoxine HCl (vitamin B ₆)	500 mg	24
H ₂ O	QSP 100 mL	—

^aSee [ref. 12](#). This solution can be stored at 4°C without autoclaving.

Table 4
Preparation of the SH3a, SH9, and ½ SH9 Media^a

Chemicals or stock	SH3a	SH9	½ SH9
N6 major salts	100 mL	100 mL	50 mL
SH minor salts	1 mL	1 mL	0.5 mL
SH vitamins	1 mL	1 mL	0.5 mL
EDFS (stock solution)	20 mL	20 mL	10 mL
Myo-inositol	100 mg	100 mg	50 mg
Sucrose	30 g	20 g	10 g
2-4 D	4 mg	—	—
BAP	0.5 mg	—	—
pH	5.8	5.8	5.8
H ₂ O	QSP 1 L	QSP 1 L	QSP 1 L
Phytigel	3 g	—	—
Kalys agar	—	7 g	7 g ^b

^aFor each medium is indicated the amount of stock solution or product required for preparing 1 L of the medium.

^bUse 9 g of Kalys agar for vertical or slant plates

2. Materials

2.1. Media

1. The preparation of the plant media stock solutions is described in [Tables 1 to 3](#).
2. The preparation of the various plant media is described in [Table 4](#).
3. The composition of the various plant media is described in [Table 5](#).
4. Solidifying agent (see [Note 1](#)):
 - a. Phytigel (Sigma, cat. no. P-8169): used for the SH3a medium.
 - b. Kalys agar HP 696-7470 (Kalys, Roubaix, France): used for the SH9 and ½ SH9 media.

Table 5
Composition of the SH3a, SH9, and ½ SH9 Media

Chemicals	SH3a	SH9	½ SH9
MgSO ₄ ·7H ₂ O	0.75 mM	0.75 mM	0.375 mM
KNO ₃	28 mM	28 mM	14 mM
(NH ₄) ₂ SO ₄	3.5 mM	3.5 mM	1.75 mM
CaCl ₂ ·2H ₂ O	1.1 mM	1.1 mM	0.55 mM
KH ₂ PO ₄	3 mM	3 mM	1.5 mM
MnSO ₄ ·H ₂ O	6 µM	6 µM	3 µM
H ₃ BO ₃	80 µM	80 µM	40 µM
ZnSO ₄ ·7H ₂ O	3.5 µM	3.5 µM	1.75 µM
KI	6 µM	6 µM	3 µM
Na ₂ MoO ₄ ·2H ₂ O	1 µM	1 µM	0.5 µM
CuSO ₄ ·5H ₂ O	0.8 µM	0.8 µM	0.4 µM
CoCl ₂ ·6H ₂ O	0.4 µM	0.4 µM	0.2 µM
Nicotinic acid	40 µM	40 µM	20 µM
Thiamine HCl (vitamin B1)	15 µM	15 µM	7.5 µM
Pyridoxine HCl (vitamin B6)	24 µM	24 µM	12 µM
EDFS (stock solution)	0.38 mM	0.38 mM	0.19 mM
Myo-inositol	0.55 mM	0.55 mM	0.275 mM
Sucrose	3%	2%	1%
2-4 D	4 mg/L (16 µM)	—	—
BAP	0.5 mg/L (2 µM)	—	—
pH	5.8	5.8	5.8

5. YEB (13, for *Agrobacterium* culture): 5 g/L Bacto beef extract, 1 g/L Bacto yeast extract, 5 g/L peptone, 5 g/L saccharose, 2 ml/L magnesium sulfate (1 M stock solution), pH 7.2. For solid medium, add 15 g/L Bacto agar before autoclaving.

2.2. Plant Material

1. *M. truncatula* (Gaertn.) line R108-1 (c3) (Fig. 1A) is described in ref. 5 and is called R108 throughout this text (see Note 2).

2.3. Agrobacterium Strain and T-DNA Vectors

1. *Agrobacterium tumefaciens* EHA105 strain (14) is recommended for R108 transformation experiments (see Note 3).
2. Conventional binary vector other than pBin19 (15) derivatives can be used for *M. truncatula* transformation experiments (see Note 4). Plasmids are introduced in the *Agrobacterium* strain by triparental mating as described (16) or by electroporation (also see Chapter 3).

2.4. Stock Solutions

2.4.1. Phytohormone Stocks

1. 2,4-Dichlorophenoxy acetic acid (2,4-D): stock solution 1 mg/mL in water. Final concentration is 4 mg/L.
2. 6-Benzylaminopurine (BAP): stock solution 1 mg/mL. First dissolve in a small volume of 2 N NaOH and bring to 1 mg/mL with water. Final concentration is 0.5 mg/L.

2.4.2. Antibiotic Stocks (see **Notes 4 and 5**)

1. Augmentin (GlaxoSmithKline, Nanterre, France): stock 0.2 g/mL in sterile water. Final concentration for media is 800 mg/L.
2. Basta™ gluphosinate-ammonium (Hoechst Schering AgrEvo GmbH, Frankfurt/Main, Germany): stock 200 g/L in 'Liberty' commercial solution. Final concentration for media is 3 mg/L.
3. Kanamycin (Sigma, cat. no. 25389-94-0): stock 40 mg/mL in sterile water. Final concentration for media is 40 mg/L.
4. Hygromycin (HygroGold™, InvivoGen, www.invivogen.com): stock 50 mg/mL. Final concentration for media is 10 mg/L.

2.5. Other Supplies

1. Sterilized or clean sand.
2. Sandpaper (commercial fine sandpaper for hard material).
3. Bayrochlor solution (BAYROL, Germany: the active compound in this product is sodium dichlorisocyanurate. Bayrochlor is an industrial disinfectant compound. Use a 7 g/L solution for seed sterilization.
4. Glass pots for in vitro culture (1 L vol with bright opening) or Magenta boxes.
5. Nutrient solution: N/P/K 18/6/26 (Soluplant, Duclos International, Lunel Viel, France).
6. Teepol L (detergent: <http://teepol.co.uk/products.html>).
7. Na-hypochlorite solution (6 or 12°C).
8. Vacuum flask (250-mL Erlenmeyer form filtration flask).
9. Vacuum pump. We have used a tap water pump and an electric pump with equal success.
10. Alimentary plastic film.
11. In vitro growth chamber lighting: we use alternating Mazdafluor Prestiflux-HF Incandia: 4A TF"P"58W/inc and Mazdafluor Blanc Industrie 33 (6J TF58W/BI) tubes for a good light spectrum.

3. Methods

As Basta selection is the most convenient one (see **Notes 4 and 5**), the protocol described below is for Basta selection.

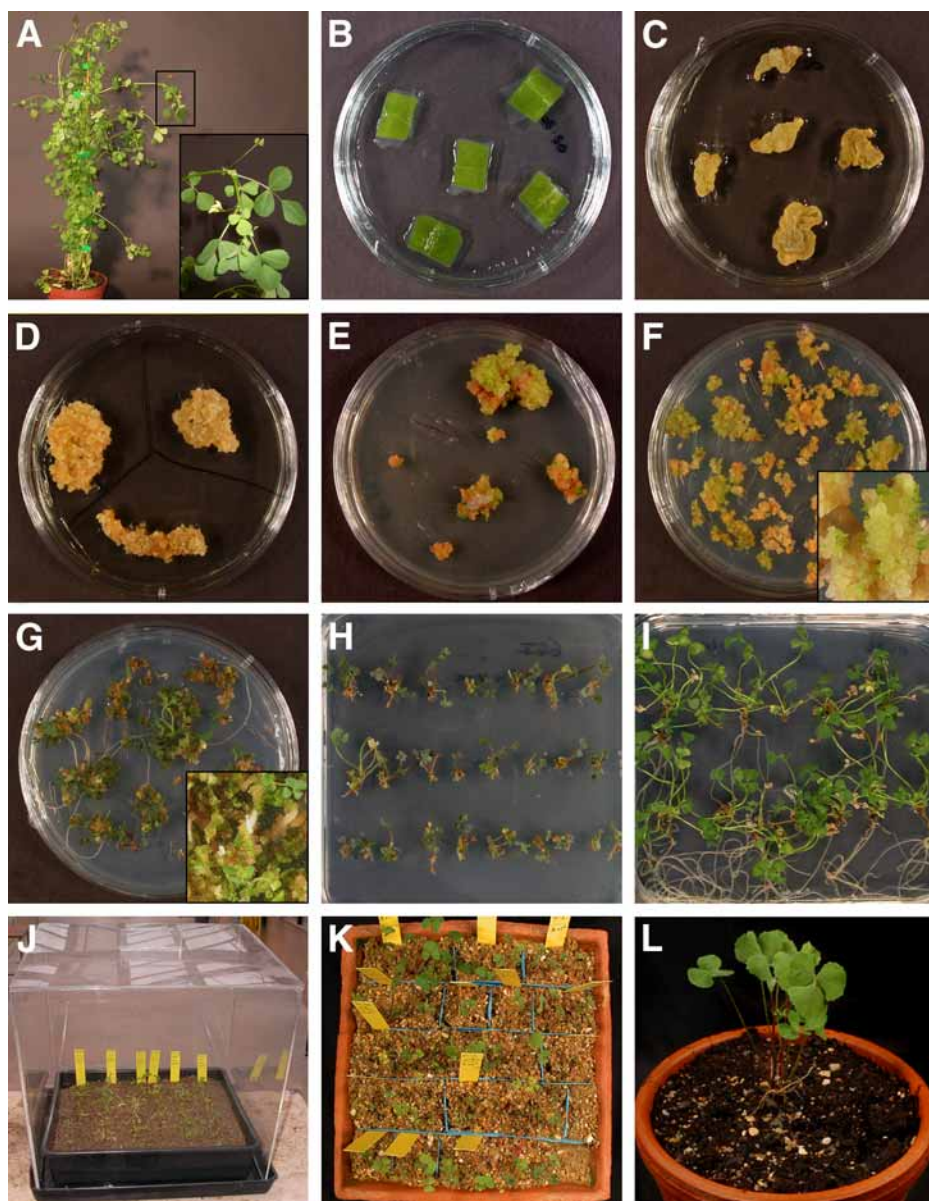


Fig. 1. Different steps of *M. truncatula* R108 regeneration. **(A)** *M. truncatula* R108 greenhouse-grown plants. Bottom right, enlargement of one shoot. **(B)** Transformed leaf explants after 2 d of co-culture on SH3a without antibiotics. Note the halo of *Agrobacterium* starting to grow around the explants. **(C)** Explants after 2 wk on SH3a callus-inducing medium. The leaf explants are deformed by the tissue proliferation (callus formation) and have lost their green color owing to growth in the dark.

3.1. Preparation of Plant Materials for Transformation

Either in vitro plants or greenhouse-grown plants can be used for transformation experiments. The growth cycle of R108 takes 6 mo from seed to seed. Growth conditions are important for the success of the transformation experiment (see **Note 6**).

3.1.1. Preparation of Plant Materials from Sterilized Seeds (see **Note 7**)

1. Seeds are scarified with sandpaper, sterilized for 30 min in a Bayrochlor solution, rinsed four times 5 min in sterile water, and germinated on sterile wet Whatmann 3MM paper in 90-mm-diameter Petri dishes, in the dark at room temperature.
2. After 2 d seedlings are transferred onto $\frac{1}{2}$ SH9 medium in 1-L glass pots (or Magenta boxes) and grown for 3 wk in the growth chamber (24°C; 16-h light) before leaf explants can be used for transformation.

3.1.2. Preparation of Plant Materials from Greenhouse-Grown Plants (see **Note 7**)

1. Seed are scarified with sandpaper and left to germinate in pure sand for 2 wk in the greenhouse (16-h day period, 60% relative humidity, 16°C at night and 22°C daily temperature with additional light: 200 $\mu\text{E}/\text{m}^2/\text{s}$). At this stage, seeds are sown at high density (one plant per cm^2) in a 10-cm-deep tray and watered with water until they are fully germinated. During this time the tray is covered with a transparent plastic dome or with plastic foil. Once the first leaf appears the plantlets are watered with nutrient solution and the tray can be open.
2. Two-weeks old plantlets with two to three leaves are then transferred to plastic pots with vermiculite or in a mixture of sand and soil (1/4:3/4) in the greenhouse

(figure caption continued from previous page) **(D)** Five-week-old calli grown on SH3a medium. Calli look like brown sugar powder. **(E)** Calli after 3 wk on SH9 medium at light. Differentiating embryos appear as green spots. At this stage calli are friable. **(F)** Calli after 4 wk on SH9 medium at light. A friable callus was spread on the medium to allow better contact between the developing embryos and the medium. Bottom right, enlargement of an embryogenic callus. Embryos are green. **(G)** Development of the embryos into plantlets after 6 wk on SH9 medium. At this stage embryos and the first plantlets are present on the plate. Bottom right, enlargement of a region with small plantlets. **(H)** The plantlets that developed on SH9 medium are transferred on $\frac{1}{2}$ SH9 square plates to allow rooting. Plates are maintained as slants to allow growth of the roots along the medium. **(I)** Growth of the plantlets after 3 wk on $\frac{1}{2}$ SH9 medium. **(J)** Adaptation of the plantlets to the greenhouse conditions. Plants are grown in sand and maintained under high humidity using a plate containing water under the recipient containing the plants and a transparent lid over it. **(K)** Three-week-old plants after their transfer to the green house. The lid was removed and plants watered with nutrient solution. **(L)** Transgenic plant in a pot with sand and soil.

(16-h day period, 60% relative humidity, 16°C at night and 22°C daily temperature with additional light: 200 $\mu\text{E}/\text{m}^2/\text{s}$) or in growth chambers (16-h day period, 60% relative humidity, 22°C temperature with 200 $\mu\text{E}/\text{m}^2/\text{s}$ light) for 3 wk before the transformation experiment (5-wk-old plants).

3. Plants are watered twice a day alternatively with nutrient solution and water (see **Note 8**).
4. The 5-wk-old plants (15 cm high, 10–20 expanded leaves) should be ready for in vitro transformation experiments.
5. Young expanded leaves should be used for the transformation experiments. These leaves are generally robust enough for the sterilization step.
6. Alternatively, entire flowers of older plants can be used (6). In this case the petals, sepals, and pistils of young open flowers are used for the transformation experiment. This allows use of greenhouse-grown plants for a longer period.

3.2. Preparation of *Agrobacterium* Culture (see Note 9)

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. Shaker incubate at 30°C (200 rpm), overnight.
2. The day before the transformation, inoculate a 250-mL flask containing 30 mL *Agrobacterium* culture (with appropriate antibiotics for selection) with 1 mL of the 2-mL overnight preculture. This culture is shaker incubated in a 250 mL Erlenmeyer flask at 30°C overnight (200 rpm). The $\text{OD}_{600\text{nm}}$ of this culture should reach 0.6 the day of the transformation.
3. Centrifuge the 30 mL *Agrobacterium* culture at 3000g for 20 min and gently resuspend the pellet in 50 mL sterile SH3a liquid medium ($\text{OD}_{600\text{nm}} = 0.6$). Then transfer the culture to a vacuum flask.

3.3. Leaf Explant Preparation and Infiltration (see Note 10)

For transformation, choose leaves from 4- to 6-wk-old plants either grown in in vitro culture or in the greenhouse. These leaves should be round and healthy, without too many hairs. Plan on five leaflets per plate. Each leaflet can generate at least one transformed plant. If you use in vitro plants, go directly to **step 4** below. After **step 3** all manipulations should be done under sterile conditions.

1. Sterilization of greenhouse-grown leaves is done in a 50-mL Falcon tube (20–30 leaves per tube). Leaflets are first rinsed in tap water containing two to three drops of Teepol. The tubes are inverted several times to wet all the leaves, and then they are rinsed with tap water until no more foam is present.
2. Replace water with Na-hypochlorite solution (6° Cl), mix gently, and leave the tube for 7 min in a rack (lid up). Return the tube (lid down, standing on the bench) and wait for an additional 7 min.
3. Under sterile conditions, rinse the leaflet three times with sterile water in the same 50-mL Falcon tube.

4. Place the sterilized leaflets into a 9-cm Petri dish with approximately 30 mL sterile water. Cut the leaflets into square pieces by removing the edges of the leaflets with a sterile scalpel.
5. Place the cut leaf pieces into the *Agrobacterium* culture (prepared in **Subheading 3.2.**) in a sterile vacuum flask (20–30 leaf pieces per 50 mL culture). Shake the flask gently to separate the leaf pieces.
6. Apply a vacuum to the leaf explants in the SH3a solution with the *Agrobacterium* for 20 min at 650 psi. To avoid cell damage, the vacuum should be released slowly.
7. Once the vacuum is released, the vacuum flask is placed on a shaking (50–60 rpm) table at room temperature for 1–2 h to allow the tissue to recover from the infiltration procedure.

3.4. Cocultivation (48 Hours)

This step allows the bacteria in contact with the plant cells to transfer the T-DNA to the plant nucleus. This is during this period of time that the transformation process occurs.

1. Under the sterile laminar flowhood, transfer the explants to an empty 9-cm Petri dish and remove most of the bacterial solution with a pipet (see **Note 11**).
2. Transfer the leaf explants to solid SH3a medium without antibiotics. The lower side (abaxial) of the leaf explants should be in contact with the medium.
3. The plates are then sealed with alimentary plastic film (see **Note 12**) and incubated for a maximum of 2 d in the dark in the plant growth culture room (24°C). During this cocultivation step care should be taken that the agrobacteria do not overgrow the leaf explants (**Fig. 1B**).

3.5. Callus Formation Step (5 to 6 Weeks)

This step will allow the transformed tissue to multiply and form calli. In addition, the hormone treatment will induce the embryogenesis process. By the end of the callus formation step, the pre-embryos should have formed.

1. The leaf explants are removed from the cocultivation medium and wiped gently on fresh solid (SH3a or SH9) medium to remove excess bacteria that have grown on the explants.
2. The leaf explants are transferred to new SH3a medium with 800 mg/L Augmentin (to eradicate the agrobacteria) and 3 mg/L Basta to select for the transformed cells if the vector confers Basta resistance. If the vector used for transformation confers kanamycin resistance, Basta is replaced by 40 mg/L kanamycin. If the vector confers hygromycin resistance, use 10 mg/L hygromycin B (see **Notes 4 and 5**).
3. Plates are sealed with alimentary plastic film and placed in the dark in the growth chamber (24°C) for 5 to 6 wk. Check plates regularly for contamination.
4. The herbicide- or antibiotic-resistant callus material (**Fig. 1C–D**) can be seen 2 wk after infiltration. These calli are transferred to new SH3a medium every 2 to 3 wk.

3.6. Embryogenesis (3 to 6 Weeks)

At this step the calli look like brown sugar powder (**Fig. 1D**) and are transferred to hormone-free medium and placed in the light. These two changes will induce embryogenesis, followed by plantlet development. The selection for the selectable marker can be maintained in the SH9 medium for the first 3 wk to reduce escapes but should be left out in subsequent subcultures because they reduce the regeneration capacity of the plant (see **Note 13**).

1. Transfer calli to SH9 medium and place them in the light ($130 \mu\text{E}/\text{m}^2/\text{s}$; in the in vitro growth chamber (24°C , 12-h photoperiod).
2. Calli are then transferred to new SH9 medium every 3 wk until the pre-embryos appear (between 3 and 6 wk on this medium). Calli at this stage should be friable and start to turn green (**Fig. 1E**).
3. From the calli, pre-embryos will develop in true embryos between 20 and 30 d after the transfer to the SH9 medium (**Fig. 1F**) (see **Note 14**).

3.7. Plantlet Development (2 to 6 Weeks)

1. About 2 to 3 wk later, plantlets start to develop from the embryos (**Fig. 1G**).
2. When plantlets are formed (**Fig. 1G**), transfer them to $\frac{1}{2}$ SH9 medium (**Fig. 1H**) to induce rooting.
3. When rooting starts, the plantlets should be transferred to $\frac{1}{2}$ SH9 square plates ($120 \times 120 \text{ mm}$; **Fig. 1H**), which will be placed vertically (or as 45° slants) in the growth chamber to allow roots to grow along the medium. (*M. truncatula* R108 roots grow poorly inside the medium; **Fig. 1I**.) In this case the amount of Kalys agar should be raised to 9 g/L to solidify the medium better. Rooting of the plantlets on this medium takes 2–6 wk.

3.8. Transfer of the Transgenic Plants to the Greenhouse

The plant material transferred from the in vitro conditions to the greenhouse is very sensitive to the change in the humidity conditions. Thus, to avoid significant loss, plants should be maintained at the beginning of the transfer in water-saturated conditions and then adapted progressively to normal greenhouse conditions.

1. Plantlets that have developed few leaves and roots on $\frac{1}{2}$ SH9 medium are transplanted into tray containing sterilized (or clean) sand covered with a transparent lid (**Fig. 1J**).
2. Plants should be watered with tap water during the first 2 wk and subsequently with nutrient solution. A plate is placed under the tray in order to keep it in water.
3. The sand should always be humid. For the first 5 d the lid is kept closed to keep the plants in an atmosphere saturated with water. Then the lid is progressively opened to reduce the humidity level slowly.
4. At the end of the second week, the lid can be completely removed (**Fig. 1K**). The plants are then watered alternately with nutrient solution and water.

5. When the plants develop new leaves under greenhouse conditions, they can be transferred to pots with a mixture of soil/sand (3:1, v/v; **Fig. 1L**).
6. We call these plants T₀ plants. If they develop normally they should flower and set seeds after 2 to 3 mo.
7. The selection for Basta-resistant transgenic plants grown in the greenhouse can be done at this stage by spraying a solution of 120 mg/L gluphosinate-ammonium on plantlets.
8. Complete development of the plant will take 4 to 6 mo. Approximately 50% of the transferred plantlets will survive the greenhouse transfer and develop into plants (see **Note 15**).

4. Notes

1. *M. truncatula* is very sensitive to the type of agar used during the experiment. We found that Phytigel works well for R108 during the callus-inducing period. For other media we use Kalys agar HP 696. Other gelling agents can induce browning and death of the explants (**6**).
2. *M. truncatula* R108 seeds for transformation experiments can be requested from Dr. P. Ratet (Pascal.Ratet@isv.cnrs-gif.fr).
3. Various laboratory-disarmed strains can be used; however, strain LBA4404 is very inefficient for transformation in *M. truncatula*.
4. We noticed that pBin19-derived vectors (**15**) give complex T-DNA integration patterns resulting in the formation of concatemers (**9**). These vectors should be avoided in order to simplify analysis of the transgenic plants. In addition, vectors conferring kanamycin resistance should also be avoided because selection for this antibiotic is not as stringent and often results in the regeneration of escaped plants (i.e., Kanamycin-sensitive plants; **10**).
5. Selection for Basta or hygromycin is more efficient with this plant line. In experiments using Basta selection, young plants can be further selected after regeneration in the greenhouse by spraying young plants (one to three leaves) twice with a gluphosinate-ammonium solution at 120 mg/L.
6. Our experience indicates that nonhealthy plants will not respond to the transformation/regeneration protocol. For example, one should avoid chemical treatments before the experiment and choose leaves without damage from insect or fungal infection.
7. We generally prefer to use greenhouse plants because they are healthier, better developed, and stronger and will tolerate the transformation procedure better.
8. Watering should be carefully controlled to avoid excess humidity. Ideally, the surface of the pots should start to dry between each watering. If the surface of the pots stays wet, to reduce pathogen invasion, it is necessary to reduce the number of waterings.
9. Addition of acetosyringone, usually used to induce T-DNA transfer, is not required for *M. truncatula* transformation.
10. This infiltration step, by introducing the agrobacteria inside the plant tissue, allows high-frequency transformation.

11. This agrobacterial culture should be sterilized with Na-hypochlorite before discarding it.
12. It is important to use this film rather than parafilm during all transformation experiments to allow gas exchanges between the plant material and the outside atmosphere.
13. If at this stage the agrobacteria start to grow again, the calli should be transferred to SH9 medium with augmentin (and Basta if necessary).
14. From each callus many embryos (5–20) can develop and give rise to transgenic siblings. Thus, to obtain independent transgenic plants, we generally keep a few embryos (5–10) at this stage and one transgenic plant per original explant at the end of the transformation experiment.
15. Sometimes plants grow poorly and produce few seeds. Normally this will change in the next generation (T_1 plants). Seed production of the T_0 plants can vary from a few pods to several hundred seeds per plant. If some plants develop leaves that seem to be larger and thicker than other plants, they may represent tetraploid plants. We have found a low percentage of tetraploid plants in our regeneration experiments. These transgenic plants are not more sensitive to fungal infection than the wild type.

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***Medicago truncatula* Transformation Using Cotyledon Explants**

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Summary

Medicago truncatula has been developed into a model species for legumes. Large numbers of ESTs have been sequenced, and sequencing of the complete gene-rich space of the genotype Jemalong A17 is in progress. By using cotyledons as explants for *Agrobacterium* infection and direct shoot formation, this protocol allows for rapid production of transgenic plants from A17 and other genotypes. Transgenic plants can be regenerated and established in the greenhouse in only 3–4 mo after *Agrobacterium*-mediated transformation. Transformation frequency is in the range of 5–12%.

Key Words: *Agrobacterium tumefaciens*; cotyledon; Jemalong A17; *Medicago truncatula*; transformation; transgenic plants.

1. Introduction

Medicago truncatula, barrel medic, is a forage crop that has been developed into a model legume. It is an omni-Mediterranean species closely related to the world's major forage legume, tetraploid alfalfa. In addition to alfalfa, it can also serve as a model organism for soybean and other economically important legumes (1). *M. truncatula* has a compact genome of approximately 470 million base pairs (Mbp), simple mendelian genetics, short seed-to-seed generation time, excellent collections of phenotypic mutants, and large collections of diverse, naturally occurring ecotypes (2,3). A number of research programs have been committed to *M. truncatula*, leading to a wealth of genomic resources. Large numbers of expressed sequence tags (ESTs) (>200,000) have been sequenced (www.tigr.org), and genespace sequencing is in progress (3). A rapid transformation system is of great importance for testing functions of genes in *M. truncatula*.

The genotype Jemalong A17 is of special interest for genetic transformation, because it has been used as the subject of most of the EST and genespace sequencing efforts. Compared with R108-1, a tissue culture-responsive genotype (4), Jemalong A17 is a recalcitrant genotype in callus culture and plant regeneration. Based on modifications of the transformation method used for white clover (5), we have been able to generate transgenic *M. truncatula* plants reproducibly. The protocol described here uses cotyledonary explants for direct shoot organogenesis bypassing the callus formation phase. The system can be used for genetic transformation of A17 and other genotypes. The transformation frequency for A17 is about 5% based on the number of plants produced and the number of explants used. Higher transformation frequency (12%) was obtained for R108-1. Transgenic plants can be regenerated and established in the greenhouse in only 3 to 4 mo after transformation.

2. Materials

2.1. Plant Material

Seeds of *Medicago truncatula* genotype Jemalong A17 (see Note 1).

2.2. *Agrobacterium tumefaciens* Strain and Selectable Marker

We used *A. tumefaciens* strain AGL-1 for infection (see Note 2) and the neomycin phosphotransferase gene (*npt2*) as a selectable marker (see Note 3).

2.3. Culture Media for *A. tumefaciens*

1. LB solid medium: LB-agar medium capsules (Qbiogene, Montreal, Canada). Autoclave, cool to about 55°C, and add selection agent for the vector.
2. LB liquid medium: LB medium capsules (Qbiogene). Autoclave, cool to about 55°C, and add selection agent for the vector.

2.4. Tissue Culture

1. Concentrated sulfuric acid.
2. Surface sterilants: 70% (v/v) ethanol and 30% (v/v) commercial Clorox bleach with 0.1% (v/v) Tween-20.
3. 6-Benzylaminopurine (BAP): 1 mg/mL stock solution (PhytoTechnology Laboratories, Shawnee Mission, KS).
4. 1-Naphthaleneacetic acid (NAA) (Sigma): prepare 2 mg/mL stock solution by dissolving the powdered chemical in a few drops of 1 M KOH and add ddH₂O to volume.
5. Acetosyringone (ACROS Organics, Morris Plains, NJ): prepare fresh 100 mM acetosyringone (3',5'-dimethoxy-4'-hydroxyacetophenone) by dissolving 0.098 g acetosyringone in 5 mL dimethyl sulfoxide (DMSO).
6. Kanamycin (PhytoTechnology Laboratories): prepare 50 mg/mL stock in ddH₂O, filter-sterilize, and store at -20°C.

7. Cefotaxime (Agri-Bio, North Miami, FL): prepare 250 mg/mL stock in ddH₂O, filter-sterilize, and store at -20°C. For rinsing infected explants, concentrated cefotaxime solution (500 mg/L) is prepared and filter-sterilized at the time of use.
8. Indole-3-butyric acid (IBA): 1 mg/mL stock solution (PhytoTechnology Laboratories).
9. Alfalfa juice: collect young leaves from the alfalfa genotype Regen-SY4D grown in the greenhouse, rinse with 70% ethanol, sterilize with 30% Clorox solution for 1 min, and then rinse three times in sterile water. Grind and extract liquid.
10. Liquid TM-1 medium: MS medium (PhytoTechnology Laboratories) supplemented with 1 mg/L BAP, 0.1 mg/L NAA, 0.40 g L-cysteine, 0.154 g dithiothreitol, 100 μ M acetosyringone, and 3% (w/v) sucrose. Adjust pH to 5.8 with KOH and filter-sterilize.
11. Solid TM-1 medium: MS medium (PhytoTechnology Laboratories) supplemented with 1 mg/L BAP, 0.1 mg/L NAA, 3% (w/v) sucrose and solidified with 0.8% (w/v) agar-agar (Sigma). Adjust pH to 5.8 with KOH, autoclave, and add 100 μ M acetosyringone.
12. TM-2 medium: MS medium (PhytoTechnology Laboratories) supplemented with 1 mg/L BAP, 0.1 mg/L NAA, 3% (w/v) sucrose and solidified with 0.8% (w/v) agar-agar (Sigma). Adjust pH to 5.8 with KOH, autoclave, and add 50 mg/L kanamycin and 250 mg/L cefotaxime.
13. TM-3 medium: MS medium (PhytoTechnology Laboratories) supplemented with 0.2 mg/L IBA, 1% (w/v) sucrose, and solidified with 0.8% (w/v) agar-agar. Adjust pH to 5.8 with KOH, autoclave, and add 50 mg/L kanamycin and 250 mg/L cefotaxime.
14. MSO medium: half-strength MS medium (PhytoTechnology Laboratories) supplemented with 1% (w/v) sucrose and solidified with 0.25% (w/v) Gelrite (Sigma). Adjust pH to 5.8 with KOH, and autoclave.
15. Autoclaved glass bottle (100 mL).
16. Autoclaved glass flask (125 mL).
17. Sterile distilled water.
18. Sterile filter paper.
19. Sterile plastic Petri dishes, parafilm.
20. 15-mL Sterile Falcon tubes.
21. Forceps, scalpel, and blades.
22. Sterile 5-cm-diameter (175-mL) plastic culture vessels (Greiner Bio-One, Longwood, FL).
23. Drummond Pipet-Aid and sterile disposable pipets.
24. Rainen Pipetman and tips.
25. Magnetic stirrer and stir bars.
26. Shaker/incubator.
27. Rotary shaker.
28. Swing rotor centrifuge.
29. Spectrophotometer.
30. Metro Mix 350 soil (Sun Gro Horticulture, Terrell, TX).

3. Methods

3.1. Seed Sterilization and Germination

1. Place seeds in a 100-mL glass bottle, add about 10 mL concentrated sulfuric acid, and stir gently for 5–8 min.
2. Rinse the seeds three times with 4°C sterile distilled water.
3. Add 25 mL 30% Clorox solution and stir for 8 min.
4. Rinse the seeds three times with 4°C sterile distilled water.
5. Plate seeds onto sterile filter paper in a Petri dish. Add sterile water to the dish to cover the seeds; the seeds should not float.
6. Place the dish in a culture room or growth chamber at 25°C in fluorescent light (40 $\mu\text{E}/\text{m}^2/\text{s}$) at a photoperiod of 16 h. Allow the seeds to germinate for 1–2 d.

3.2. *Agrobacterium* Preparation

1. Streak *A. tumefaciens* from a glycerol stock onto an 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 15-mL sterile tube containing 5 mL LB medium with antibiotic selection appropriate for the vector used. Incubate the cultures on a shaker/incubator at 250 rpm at 28°C for about 8 to 10 h.
3. Transfer 1 mL of the *Agrobacterium* cultures into a flask containing 50 mL LB medium with antibiotic selection appropriate for the vector used. Incubate the cultures on a shaker/incubator at 250 rpm at 28°C overnight, until the cultures have reached an OD_{600} of about 1.0.
4. Centrifuge the *Agrobacterium* cultures at 2400g for 10 min.
5. Pour off supernatant, resuspend the pellet with liquid TM-1 medium, and adjust the OD_{600} to 0.4 to 0.5.

3.3. Explant Preparation

1. Remove seed coat of the germinated seeds with forceps.
2. Cut hypocotyls from the cotyledons; leave about 1.0 mm of hypocotyl stalk on the cotyledons (**Fig. 1A**).
3. Separate the two cotyledons of a seed precisely, with a scalpel (**Fig. 1A**).
4. Transfer the cut cotyledons into a Petri dish containing diluted *Agrobacterium* cultures ($\text{OD}_{600} = 0.1$).
5. Continue, and collect all cotyledons (see **Note 4**).

3.4. Inoculation of Explants and Cocultivation

1. Pipet off the diluted *Agrobacterium* solution and pour *Agrobacterium* cultures ($\text{OD}_{600} = 0.4$ to 0.5, about 25 mL) onto the explants in the Petri dish. Add 0.5 mL of alfalfa extracts to the dish.
2. Seal the dish with parafilm and incubate for 30 min on a rotary shaker at 30 rpm.
3. Place a sterile filter paper in another Petri dish.

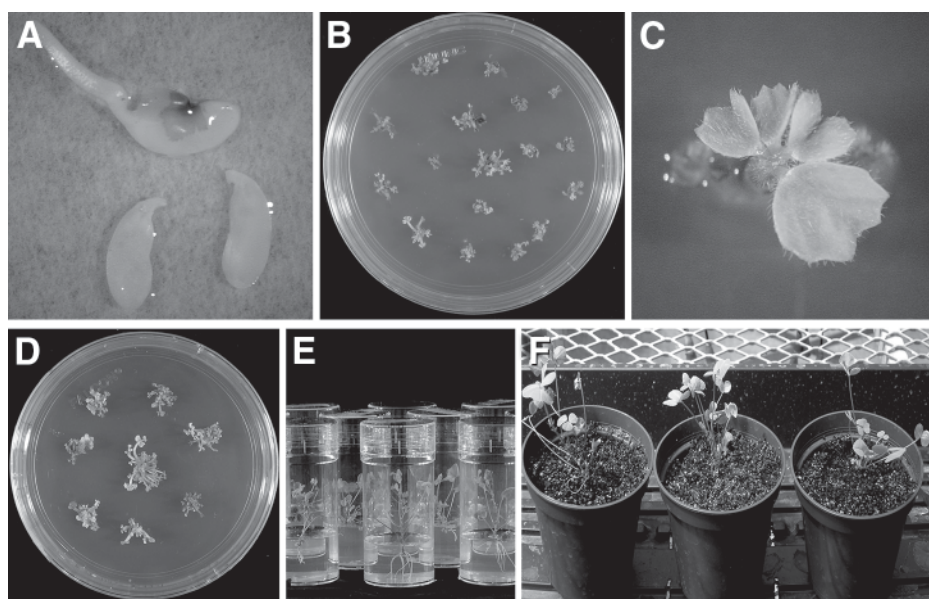


Fig. 1. Transgenic *Medicago truncatula* plants obtained after *Agrobacterium tumefaciens*-mediated transformation of cotyledonary explants. **(A)** Germinated seed and dissected cotyledons. **(B,C)** Shoots formed after 3 wk of selection. **(D)** Shoots obtained after 6 wk of selection. **(E)** Rooted plantlets 11 wk after cotyledon transformation. **(F)** Transgenic *M. truncatula* plants grown in the greenhouse.

4. Pipet off *Agrobacterium* culture and place explants on the sterile filter paper; let the explants partially dry.
5. Transfer the cotyledons onto solid TM-1 medium.
6. Seal the dishes with parafilm and cocultivate for 2 d at 25°C in fluorescent light (40 $\mu\text{E}/\text{m}^2/\text{s}$) under a 16/8-h photoperiod.
7. After cocultivation, remove the explants from the Petri dish, rinse with concentrated cefotaxime solution (500 mg/L), and dry on sterile filter paper.

3.5. Selection and Plant Regeneration

1. Transfer the cotyledons to TM-2 medium with a selection agent (kanamycin).
2. Seal the dishes with parafilm and keep at 25°C in fluorescent light (40 $\mu\text{E}/\text{m}^2/\text{s}$) at a photoperiod of 16 h in a growth room.
3. Transfer the cotyledons every 2 wk onto fresh TM-2 medium.
4. After 4 to 6 wk, transfer the regenerated shoots (**Fig. 1B** and **C**) onto TM-3 medium.
5. After 2 to 3 wk, transfer the shoots (**Fig. 1D**) onto rooting medium MSO.
6. After roots have developed (normally 2–4 wk; **Fig. 1E**), the plantlets are ready to be transferred to soil.

3.6. Greenhouse Care 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 5**) and grow them in growth chambers (260 $\mu\text{E}/\text{m}^2/\text{s}$, 16-h d/8-h night at 24°C/20°C) for about 2 wk. Cover the flat with a plastic dome. Water the plants once a day or as needed.
2. Transfer the established plants to 4.5-inch pots filled with Metro Mix 350 soil and grow them under greenhouse conditions (390 $\mu\text{E}/\text{m}^2/\text{s}$, 16-h d/8-h night at 24°C/20°C; **Fig. 1F**; see **Note 6**). Plants can be grown on Ebb-Flo™ benches and watered three times a week with fertilized water containing 50 ppm N. (Peters Professional 15-30-15 Hi-Phos Special is used as the water-soluble fertilizer.)
3. Plants normally flower 1 mo after transplanting to the greenhouse. Mature seeds can be harvested 3 mo after transplanting the plants to the greenhouse (see **Note 7**). About 1500 seeds can easily be obtained from one transgenic plant.

4. Notes

1. Other *M. truncatula* genotypes can also be transformed with this protocol. The tissue culture-responsive genotype R108-1 had a higher transformation frequency than A17.
2. Other *Agrobacterium* strains, EHA105 and C58C1, have also been successfully used for cotyledon transformation.
3. The use of the phosphinothricin acetyltransferase (*bar*) gene and PPT selection also allowed the recovery of transgenic plants.
4. Try to collect cotyledons over a short period. Transformation efficiency will be reduced if the cotyledons are left too long in water or diluted *Agrobacterium* solution.
5. Before transfer to soil, rinse the roots with water or remove excessive medium with a damp paper towel.
6. The plants can also be transferred to bigger pots for more seed production.
7. *M. truncatula* is an inbreeding species; no cross pollination is needed for seed production.

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***Medicago truncatula* Transformation Using Root Explants**

Cynthia Crane, Richard A. Dixon, and Zeng-Yu Wang

Summary

Medicago truncatula is a forage crop that has been developed into a model legume. As a model plant, *M. truncatula* is particularly useful for the study of root endosymbiotic associations, including nodulation and mycorrhizal colonization. The development of different transformation methods is an important aspect for functional genomic studies in the species. This protocol describes an efficient system for generating transgenic plants from *M. truncatula* roots based on *Agrobacterium tumefaciens*-mediated transformation. Furthermore, the protocol can be easily adapted for recovering fertile transgenic plants from transformed hairy roots.

Key Words: *Agrobacterium tumefaciens*; hairy root; *Medicago truncatula*; root; transformation; transgenic plants.

1. Introduction

Medicago truncatula has been chosen as a model legume species in view of its small, diploid genome, self-fertility, and short life cycle (1,2). As a legume, and unlike the most studied model plant, *Arabidopsis*, *M. truncatula* establishes symbiotic relationships with nitrogen-fixing bacteria (Rhizobia). In addition, roots of *M. truncatula* are also colonized by beneficial arbuscular mycorrhizal fungi (1). Transgenic *M. truncatula* plants have been obtained by *Agrobacterium tumefaciens*-mediated transformation using leaf or cotyledon as explants (3). *Agrobacterium rhizogenes*-mediated transformation allowed the production of composite plants (a term derived from the fact that transformed roots are induced on a nontransformed plant), in which transformed hairy roots have been used as a powerful tool for studying endosymbiotic associations (4).

We have developed a protocol for producing transgenic *M. truncatula* plants by *Agrobacterium tumefaciens*-mediated transformation of roots. The regeneration procedure from root-derived calli can be easily applied to the regeneration of fertile plants from transformed hairy roots (see **Note 1**).

2. Materials

2.1. Plant Material

Seeds of *Medicago truncatula* genotype R108-1 (5).

2.2. Agrobacterium Strains and Selectable Marker

For root transformation, we used *Agrobacterium tumefaciens* strain EHA105 (see **Note 2**) for infection and the phosphinothricin acetyltransferase gene (*bar*) as the selectable marker (see **Note 3**).

2.3. Culture Media for Agrobacterium tumefaciens

1. LB solid medium: LB-agar medium capsules (Qbiogene, Montreal, Canada). Autoclave, cool to about 55°C, and add selection agent for the vector.
2. LB liquid medium: LB medium capsules (Qbiogene). Autoclave, cool to about 55°C, and add selection agent for the vector.

2.4. Tissue Culture

1. Concentrated sulfuric acid.
2. Surface sterilants: 70% (v/v) ethanol and 30% (v/v) commercial Clorox bleach with 0.1% (v/v) Tween-20.
3. 2,4-Dichlorophenoxy-acetic acid (2,4-D): prepare 1 mg/mL stock by dissolving the powdered chemical in a few drops of 1 M KOH and add ddH₂O to volume.
4. 6-Benzylaminopurine (BAP): 1 mg/mL stock solution (PhytoTechnology Laboratories, Shawnee Mission, KS).
5. Acetosyringone (ACROS Organics, Morris Plains, NJ): prepare fresh 100 mM acetosyringone (3',5'-dimethoxy-4'-hydroxyacetophenone) by dissolving 0.098 g acetosyringone in 5 mL dimethyl sulfoxide (DMSO).
6. Phosphinothricin (glufosinate; PPT; Sigma-Aldrich, Seelze, Germany): prepare 10 mg/mL stock in ddH₂O, filter-sterilize, and store at -20°C.
7. Cefotaxime (Agri-Bio, North Miami, FL): prepare 250 mg/mL stock in ddH₂O, filter-sterilize, and store at -20°C.
8. MSO medium: MS medium (PhytoTechnology Laboratories) with 1% (w/v) sucrose and solidified with 0.25% (w/v) Gelrite (Sigma, St. Louis, MO). Adjust pH to 5.8 with KOH and autoclave.
9. Liquid MTR-1 medium: MS medium (PhytoTechnology Laboratories) supplemented with 5 mg/L 2,4-D, 0.5 mg/L BAP, 3% (w/v) sucrose, and 100 µM acetosyringone. Adjust pH to 5.8 with KOH, and filter-sterilize.

10. Solid MTR-1 medium: MS medium (PhytoTechnology Laboratories) supplemented with 5 mg/L 2,4-D, 0.5 mg/L BAP, 3% (w/v) sucrose and solidified with 0.8% (w/v) agar-agar (Sigma). Adjust pH to 5.8 with KOH, autoclave, and add 100 μ M acetosyringone.
11. MTR-2 medium: MS medium (PhytoTechnology Laboratories) supplemented with 5 mg/L 2,4-D, 0.5 mg/L BAP, 3% (w/v) sucrose and solidified with 0.8% (w/v) agar-agar. Adjust pH to 5.8 with KOH, autoclave, and add 5.0 mg/L PPT and 250 mg/L cefotaxime.
12. MTR-3 medium: MS medium (PhytoTechnology Laboratories) with 2% (w/v) sucrose and solidified with 0.8% (w/v) agar-agar. Adjust pH to 5.8 with KOH, autoclave, and add 2.5 mg/L PPT and 250 mg/L cefotaxime.
13. Autoclaved glass bottle (50 mL).
14. Autoclaved glass flask (125 mL).
15. Sterile distilled water.
16. Sterile filter paper.
17. Sterile plastic Petri dishes, parafilm.
18. 15-mL Sterile Falcon tubes.
19. Forceps, scalpel, and blades.
20. Sterile 5.0-cm-diameter (175 mL) plastic culture vessels (Greiner Bio-One, Longwood, FL).
21. Drummond Pipet-Aid and sterile disposable pipets.
22. Rainen Pipetman and tips.
23. Magnetic stirrer and stir bars.
24. Shaker/incubator.
25. Rotary shaker.
26. Swing rotor centrifuge.
27. Spectrophotometer.
28. Metro Mix 350 soil (Sun Gro Horticulture, Terrell, TX).

3. Methods

3.1. Seed Sterilization and Germination

1. Place seeds in a 50 mL autoclaved glass bottle, add about 5 mL concentrated sulfuric acid, and stir gently for 5 to 8 min.
2. Rinse the seeds three times with 4°C sterile distilled water.
3. Add about 10 mL 30% Clorox solution and stir for 8 min.
4. Rinse the seeds three times with 4°C sterile distilled water.
5. Plate seeds onto sterile filter paper in a Petri dish. Add sterile water to the dish to cover the seeds; the seeds should not float. Place the dish in the dark overnight.
6. Place the dish in a culture room or growth chamber at 25°C in fluorescent light (40 μ E/m²/s) at a photoperiod of 16 h. Allow the seeds to germinate for 3 to 4 d.
7. Transfer germinated seeds to plastic vessels containing MSO medium and grow them for 3 or more weeks until enough roots are formed (see **Note 4**).

3.2. *Agrobacterium* Preparation

1. Streak *A. tumefaciens* from a glycerol stock onto an 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 15-mL sterile tube containing 5 mL LB medium with antibiotic selection appropriate for the vector used. Incubate the cultures on a shaker/incubator at 250 rpm at 28°C for about 8 to 10 h.
3. Transfer 1 mL of the *Agrobacterium* cultures into a flask containing 50 mL LB medium with antibiotic selection appropriate for the vector used. Incubate the cultures on a shaker/incubator at 250 rpm at 28°C overnight, until the cultures have reached an OD₆₀₀ of about 1.0.
4. Centrifuge the *Agrobacterium* cultures at 2400g for 10 min.
5. Pour off the supernatant, resuspend the pellet with liquid MTR-1 liquid medium, and adjust the OD₆₀₀ to 0.4 to 0.5. Transfer the *Agrobacterium* cultures to a Petri dish.

3.3. *Explant Preparation, Inoculation of Explants, and Cocultivation*

1. Carefully remove plants from the MSO medium and clean off excess Gelrite from the roots. Place the root in a Petri dish and cut the roots into approx 1-cm segments.
2. Transfer the root segments to the Petri dish containing *Agrobacterium* cultures.
3. Seal the dish with parafilm and incubate for 30 min on a rotary shaker at 30 rpm.
4. Pipet off the *Agrobacterium* solution and place explants onto sterile filter paper in another dish; let the explants partially dry.
5. Transfer root segments onto solid MTR-1 medium (**Fig. 1A**).
6. Seal the dishes with parafilm and cocultivate for 2 d in the dark at 25°C.

3.4. *Selection and Plant Regeneration*

1. Transfer the root segments to MTR-2 medium with selection agent.
2. Seal the dishes with parafilm and keep in the dark at 25°C.
3. Transfer the root segments every 2 to 3 wk onto fresh MTR-2 medium. Keep the cultures in the dark. Callus will be formed on MTR-2 medium (**Fig. 1B** and **C**).
4. After 4 to 6 wk, transfer the calli onto MTR-3 medium and grow them under fluorescent light (40 $\mu\text{E}/\text{m}^2/\text{s}$) at a photoperiod of 16 h.
5. Transfer the calli every 2 to 3 wk onto fresh MTR-3 medium. Shoots will be formed on MTR-3 medium (**Fig. 1D–F**).
6. After shoots are produced (about 6 wk), transfer the shoots to MSO medium.
7. After roots are developed (normally 4–6 wk, **Fig. 1G**), the plantlets are ready to be transferred to soil.

3.5. *Greenhouse Care and Seed Harvest*

1. Transfer well-rooted plantlets to 3 $\times$ 3-inch wells in an 18-well flat (6 $\times$ 3 wells) filled with Metro Mix 350 soil (see **Note 5**) and grow them in growth chambers (260 $\mu\text{E}/\text{m}^2/\text{s}$, 16-h d/8-h night at 24°C/20°C) for about 2 wk. Water the plants once a day or as needed.

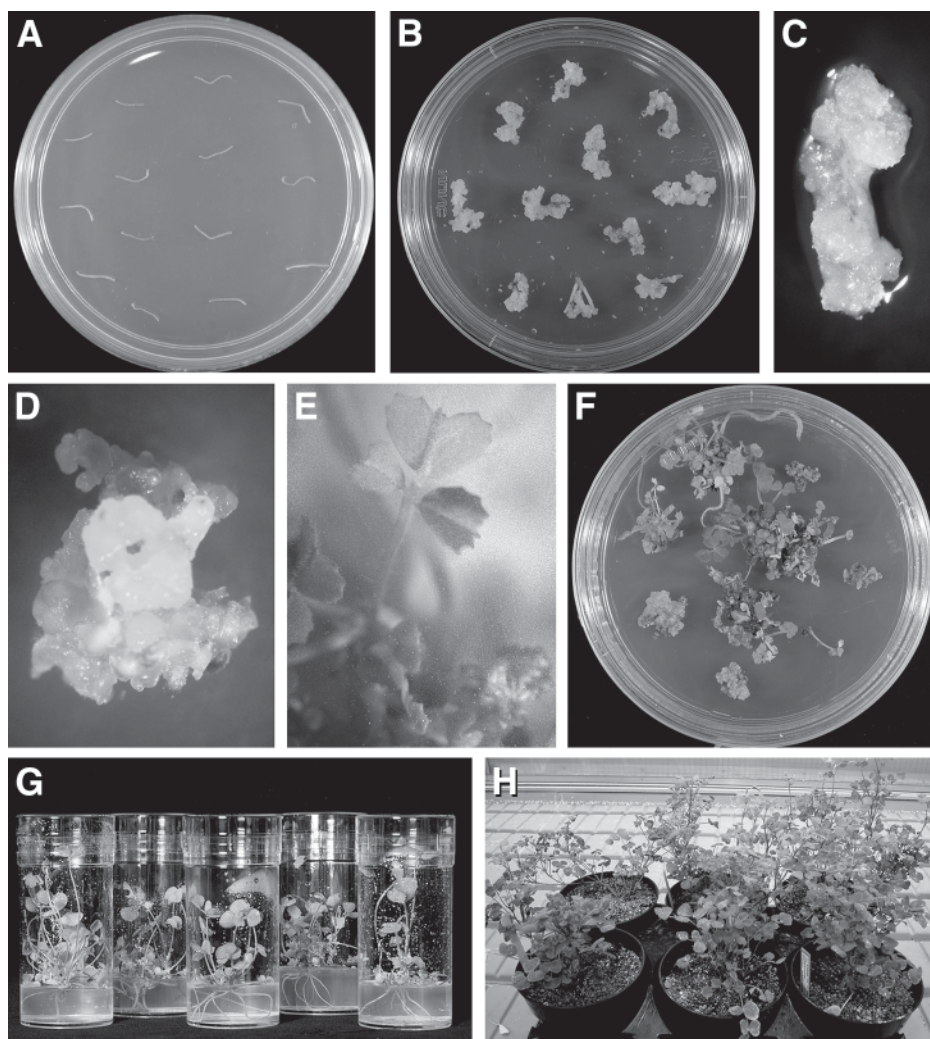


Fig. 1. Transgenic *Medicago truncatula* plants obtained after *Agrobacterium tumefaciens*-mediated transformation of roots. **(A)** Root segments after cocultivation with *Agrobacterium tumefaciens*. **(B,C)** Calli formed from root segments after *Agrobacterium*-mediated transformation and PPT selection. **(D–F)** Shoot formation after transfer of the root-derived resistant calli onto regeneration medium. **(G)** Rooted transgenic *M. truncatula* plantlets. **(H)** Greenhouse-grown transgenic *M. truncatula* plants.

2. Transfer the established plants to 4.5-inch pots filled with Metro Mix 350 soil and grow them under greenhouse conditions (390 $\mu\text{E}/\text{m}^2/\text{s}$, 16-h d/8-h night at 24°C/20°C; **Fig. 1H**; see **Note 6**). Plants can be grown on Ebb-Flo™ benches and watered three times a week with fertilized water containing 50 ppm N. (Peters Professional 15-30-15 Hi-Phos Special is used as the water-soluble fertilizer.)
3. Plants normally flower 1 mo after transplanting to the greenhouse. Mature seeds can be harvested 3 mo after transplanting the plants to the greenhouse (see **Note 7**). About 1500 seeds can be easily obtained from one transgenic plant.

4. Notes

1. Transformed hairy roots can be obtained by *Agrobacterium rhizogenes*-mediated transformation (**4**). To regenerate transgenic plants from the hairy roots, cut the hairy roots into ~1-cm segments, place the segments onto MTR2 medium, and follow the steps in **Subheading 3.4.** of this protocol.
2. Other *Agrobacterium* strains (AGL-1, C58C1, and LBA4404) can also be used for root transformation.
3. The use of the neomycin phosphotransferase gene (*npt2*) and kanamycin selection also allowed recovery of transgenic plants.
4. Roots from 3-wk to 3-mo-old plants can be used as explants for transformation.
5. Before transfer to soil, rinse the roots with water or remove excessive agar with a damp paper towel.
6. The plants can also be transferred to bigger pots for more seed production.
7. *M. truncatula* is an inbreeding species; no cross-pollination is needed for seed production.

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Nicotiana (*Nicotiana tobaccum*, *Nicotiana benthamiana*)

Tom Clemente

Summary

Agrobacterium-mediated transformation of *Nicotiana* species, namely, *Nicotiana tobaccum* and *Nicotiana benthamiana*, using leaf disks as the target explant has provided the plant community with a valuable tool for rapid evaluation of transgenes in higher plants. This protocol has a number of desirable attributes: readily available explant material, high efficiency, and a relatively quick turnaround time. The *in vitro* regeneration scheme of the leaf disks is prolific and follows an indirect organogenic differentiation. *N. tobaccum* and *N. benthamiana* are highly susceptible to a variety of disarmed *A. tumefaciens* strains, and the transformation system is amenable to a number of selection agents. This protocol has proved to be easily transferable to a point at which a single investigator with minimal training can generate thousand of events per year. Moreover, seed size and quantity per event permit monitoring of segregation in Petri plates, and sufficient biomass can be accrued from an individual plant, which can be either clonally propagated or allowed to self-pollinate or easily outcrossed.

Key Words: *npt II*; BAP; NAA; organogenesis; genetic engineering; *Agrobacterium*.

1. Introduction

Nicotiana tobaccum and *N. benthamiana* have served as model species for the plant sciences for more than 20 yr. The latter possesses the unique trait of being susceptible to a vast number of plant viruses (>500), even to those that are typically restricted to monocot hosts and, most surprisingly, even to several animal viruses (1). Consequently *N. benthamiana* has become a cornerstone for the study of host/virus interactions in plants. A criterion for a plant species to fall under the category of “model” is the ability to modulate gene expression rapidly and efficiently via plant genetic engineering. The seminal report describing a straightforward means to deliver transgenes into members of the Solanaceae family was communicated in the mid-1980s by Horsch et al. (2).

This protocol has passed the test of time and remains a viable and efficient means to generate transgenic events from both *N. tabaccum* and *N. benthamiana*. Moreover, *Agrobacterium*-based gene delivery has advanced to where transient gene expression in *Nicotiana* can be exploited to modulate gene expression temporally *in planta* (3,4) thereby complementing the use of this powerful tool for functional genomics.

The *Agrobacterium*-mediated transformation protocol relies on an indirect organogenic culture regime. This implies that differentiation of the explant first proceeds to a dedifferentiated callus phase prior to shoot organogenesis. The explant of choice is leaf disks isolated from 2–3-mo-old seedlings, although leaf disks obtained from older plants will suffice. Cell differentiation and transformation occurs about the parameter (cut edges) of the disks. Typically, stable callus proliferation can be observed within 2 wk of selection and shoot organogenesis by 4 wk of culture. The derived shoots can be rooted within a 2-wk period and acclimated to the greenhouse. Total time from explant inoculation to establishing a primary transformant (T_0) in the greenhouse is 2 to 3 mo. The T_0 plant will be hemizygous for the T-DNA. This time frame mirrors that of the *Arabidopsis* floral dip method (5,6), but the *Nicotiana* protocol necessitates additional labor and space requirements.

This transformation protocol is amenable to a number of selectable marker genes/selection agent combinations including but not limited to *npt II*/kanamycin (7), *hpt*/hygromycin (8), *bar*/glufosinate (9), and *pmi*/mannose (10). Moreover, most genotypes on *N. tabaccum* are suitable; however, the workhorse genotype for researchers has been ‘Xanthi’.

Plant genetic engineering protocols can be partitioned into two broad parameters, with the exception of the *Arabidopsis* floral dip method: (1) integration of the foreign allele into a cell and (2) differentiation of that cell to a lineage that will be transmitted to the next generation. The probability of recovering a functional transformant is equivalent to these two independent events occurring. Hence, a plant species with high *in vitro* capacity that is hypersusceptible to *A. tumefaciens* will translate to high transformation efficiency, which is calculated on a transformant in soil per explant inoculated basis. This is assuming that the cells competent for *in vitro* morphogenesis are also susceptible to *A. tumefaciens* infection. With respect to *N. tabaccum* and *N. benthamiana*, both can be classified as species with high *in vitro* capacitance and high susceptibility to *A. tumefaciens*; hence they possess a relatively high transformation frequency. The ability to generate a large quantity of transformants is desirable. However, the output potential of a plant genetic engineering protocol cannot compromise on quality, since the labor and space costs associated with screening poor-quality events may outweigh the benefit of a protocol with a high transformation frequency. The attributes, regenera-

tion potential, and *A. tumefaciens* susceptibility translate to high output rates of transgenic events with both *Nicotiana* species, but a significant portion of the derived events possess T-DNA insertions at multiple loci, which may lead to gene silencing (11). This potential problem can be alleviated by utilizing a less virulent strain of *A. tumefaciens* such as the disarmed octopine strain LBA4404 (12).

The tobacco transformation frequency using the leaf disk protocol described here is relatively efficient. Genotypes of *N. tabaccum* will display a transformation frequency ranging from 50 to 80%; frequencies with *N. benthamiana* will be significantly lower, approximately 20–30%.

2. Materials

2.1. Plant Materials

1. Seed of *N. benthamiana* is indigenous to Australia. The species probably arose from a hybridization of two progenitors, *N. suaveolens* and *N. debneyi*.
2. Seed of *N. tabaccum* genotypes reported in the literature include SR-1, Petit Havana, and Xanthi. Although some genotype variation will exist in all genetic engineering protocols, most *N. tabaccum* genotypes are suitable for transformation.

2.2. Agrobacterium Strain and Constructs

1. *A. tumefaciens* strains: LBA4404/pAL4404 (12), C58C1/pPM90 (13), EHA101/pTiEHA101 (14), EHA105/pTiEHA105 (15), and NTL₄/pTiEHA105 (16).
2. Binary vectors: selection of an appropriate binary vector will depend on the inherent antibiotic resistance of the selected *A. tumefaciens* strain (see **Note 1**). The small family of binary plasmids designated pPZP assembled by Hajdukiewicz et al. (17) constitute an excellent set of standard binary plasmids for use in *Agrobacterium*-mediated transformation. The backbone of the pPZP binary family also served as the foundation for the assembly of the well-distributed pCambia vectors (www.cambia.org). In addition, Hellens et al. (18) wrote a short guide to disarmed *A. tumefaciens* strains and commonly used binary plasmids. This report summarizes the origin and antibiotic resistance of the respective biological materials.

2.3. Stock Solutions

Major and minor stock solutions along with chelated iron stocks should be kept at 4°C. All growth regulator stocks made fresh monthly can be kept at 4°C. Alternatively, they can be stored at –20°C. Prepared media plates can be stored at 4°C for up to 1 mo.

1. MS major salts (10X; 19), per liter: 19.0 g/L KNO₃, 16.5 g/L NH₄NO₃, 3.7 g/L MgSO₄·7H₂O, 4.4 g/L CaCl₂·2H₂O, and 1.7 g/L KH₂PO₄ (see **Note 2**).
2. MS minor salts (100X; 19), per liter: 2.2 g/L MnSO₄·4H₂O, 83 mg/L KI, 620 mg/L H₃BO₄, 860 mg/L ZnSO₄·7H₂O, 2.5 mg/L CuSO₄·5H₂O, 25 mg/L NaMoO₄·2H₂O, and 2.5 mg/L CoCl₂·6H₂O.

3. Iron stock (200X).
 - a. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ stock: dissolve 5.6 g of chemical in 1 L of dH_2O . Keep the $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ container wrapped with aluminum foil.
 - b. 0.5 M Na_2EDTA stock (pH 8.0): to prepare, add 186.1 g of Na_2EDTA to 500 mL of distilled water. Monitor the pH while slowly adding pellets of NaOH . The Na_2EDTA will begin to dissolve as the pH approaches 8.0. Bring the final volume up to 1 L with distilled water.Store the $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and Na_2EDTA in separate containers at 4°C .
4. Vitamin B_5 stock (100X; 20), per liter: 10 g/L myo-inositol, 100 mg/L nicotinic acid, 100 mg/L pyridoxine-HCl, and 1.0 g/L thiamine-HCl.
5. Growth regulator stocks (see Note 3).
 - a. Naphthalene acetic acid (NAA): 1 mg/mL.
 - b. Benzylaminopurine (BAP): 1 mg/mL.
 - c. *p*-Chloro-phenoxy acetic acid (pCPA): 1 mg/mL.
6. Antibiotic stocks (see Note 4).
 - a. Carbenicillin: 50 mg/mL.
 - b. Cefotaxime: 100 mg/mL.

2.4. Media

1. YEP medium (see Note 5): 10 g/L peptone, 5 g/L yeast extract, 5 g/L NaCl (pH 7.0). Sterilize by autoclaving. Add appropriate antibiotics when cooled to 55°C .
2. Preculture medium (1 L; see Note 6): 100 mL of major salt stock, 10 mL of minor salt stock. 10 mL chelated iron prepared separately by mixing 5 mL of FeSO_4 stock and 5 mL Na_2EDTA stock and heat until solution turns yellow. Add 30 g of sucrose. Adjust pH of the medium with 1 N KOH to 5.7. Bring the medium up to 975 mL and add 8 g of grade A agar (Sigma). Sterilize by autoclaving.

Prepare vitamin and growth regulator solution. In 10 mL ddH_2O , add 10 mL vitamin B_5 stock, 1 mL BAP stock, 0.1 mL NAA stock, and 8 mg pCPA. Bring vitamin/growth regulator cocktail up to 25 mL with water and filter-sterilize. Add the sterilized vitamin/growth regulator cocktail to the autoclaved components once the temperature has reached 55°C .
3. Coculture medium (liquid 1 L; see Note 7): 10 mL of major salt stock, 1.0 mL of minor salt stock, 10 mL chelated iron prepared separately by mixing 0.5 mL of FeSO_4 stock and 0.5 mL Na_2EDTA stock and heat until solution turns yellow. Add 30 g of sucrose and 3.7 g 2-morpholinoethanesulfonic acid (MES) buffer (pH 5.4). Bring the medium up to 950 mL.

Prepare vitamin and growth regulator solution. In 10 mL of ddH_2O , add 10 mL vitamin stock, 1 mL BAP stock, 0.1 mL NAA stock, and 8 mg of pCPA. Dissolve 38 mg of acetosyringone. Bring vitamin/growth regulator cocktail up to 50 mL with water and filter-sterilize. Add the sterilized vitamin/growth

regulator cocktail to the autoclaved components once the temperature has reached 55°C.

4. Selection medium (1 L; see **Note 8**): 100 mL of major salt stock, 10 mL of minor salt stock, 10 mL chelated iron prepared separately by mixing 5 mL of FeSO_4 stock and 5 mL Na_2EDTA stock and heat until solution turns yellow. Add 30 g of sucrose. Adjust pH of the medium with 1 N KOH to 5.7. Bring the medium up to 975 mL and add 8 g of grade A agar (Sigma). Sterilize by autoclaving.

Prepare vitamin and growth regulator solution. In 10 mL ddH₂O add 10 mL vitamin B₅ stock, 1 mL BAP stock, 0.1 mL NAA stock. Add 2 mL carbenicillin stock and 1 mL of cefotaxime stock. Bring vitamin/growth regulator cocktail up to 25 mL with water and filter-sterilize. If using *nptII* add 150 mg/L kanamycin. If using *hpt* add 10 mg/L hygromycin. If using *bar* add 3 mg/L glufosinate. Add the sterilized vitamin/growth regulator cocktail to the autoclaved components once the temperature has reached 55°C.

5. Rooting medium (1 L; see **Note 9**): 50 mL of major salt stock, 5 mL of minor salt stock, 10 mL chelated iron prepared separately by mixing 5 mL FeSO_4 stock and 5 mL Na_2EDTA stock and heat until solution turns yellow. Add 10 g sucrose. Adjust pH of the medium with 1 N KOH to 5.7. Bring the medium up to 975 mL and add 8 g of grade A agar (Sigma). Sterilize by autoclaving.

Prepare vitamin and growth regulator solution. In 10 mL ddH₂O add, 10 mL vitamin B₅ stock, 0.1 mL of NAA stock. Add 2 mL carbenicillin stock and 1 mL of cefotaxime stock. Bring vitamin/growth regulator cocktail up to 25 mL with water and filter-sterilize. If using *nptII* add 75 mg/L kanamycin. If using *hpt* add 5 mg/L hygromycin. If using *bar* add 3 mg/L glufosinate. Add the sterilized vitamin/growth regulator cocktail to the autoclaved components once the temperature has reached 55°C

6. Germination medium (1 L): 100 mL of major salt stock, 10 mL of minor salt stock, 10 mL chelated iron prepared separately by mixing 5 mL FeSO_4 stock and 5 mL Na_2EDTA stock and heat until solution turns yellow. Add 30 g of sucrose. Adjust pH of the medium with 1 N KOH to 5.7. Bring the medium up to 975 mL and add 8 g of grade A agar (Sigma). Sterilize by autoclaving.

Prepare vitamin solution by taking 10 mL of vitamin B₅ stock and bringing it up to 25 mL with water. Filter-sterilize and add to the autoclaved components once the temperature has reached 55°C.

2.5. Other Supplies

1. Sundae cup vessels: Sweetheart™ lids, cat. no. LDS58; base, cat. no. DSD8X.
2. Jiffy™-cup: model 122 (Hummert™ International, Earth City, MO).
3. Soil: Metro-Mix 360 (Hummert International).
4. Magenta™ vessel: cat. no. V8505 (Sigma).
5. Hoagland's solution: cat. no. H2395 (Sigma).
6. Whatman filter paper: cat. no. 1001070 (VWR).

3. Methods

3.1. Plant Material Preparation (see Note 10)

3.1.1. From Greenhouse-Grown Plants

1. Select 4- to 6-inch (*N. benthamiana* leaves are typically smaller than *N. tabaccum*) leaves from 1- to 2-mo-old *N. benthamiana* and *N. tabaccum* plants growing under greenhouse conditions. The upper two fully expanded leaves are sufficient for 30 to 40 leaf disks.
2. Place the selected leaves in a large beaker and cover with distilled water for 20 to 30 min.
3. Drain the water and cover the leaves with 10% commercial bleach (0.6% sodium hypochlorite) supplemented with 1 drop of Tween-20 per 50 mL. Allow the leaves to soak in the bleach solution for 10 min (see **Note 11**). Rinse five times with sterile distilled water.

3.1.2. From Seeds

1. Place approximately 50 μ L packed volume of seeds from the respective species in an Eppendorf tube, sterilized by exposure to 1 mL of 100% ethanol for 1 min.
2. Following the ethanol wash, briefly centrifuge for at 16,000g for 15 s.
3. Remove ethanol and replace with 1 mL 50% commercial bleach (3% sodium hypochlorite) supplemented with Tween-20 (1 drop per 50 mL). Vortex briefly. Let the seed sit in the bleach solution for 15 min.
4. Centrifuge the seed at 16,000g for 30 s and draw off bleach with a pipet.
5. Wash the sterilized seed with sterile distilled water five times by repeating **step 4**.
6. Transfer the sterilized seeds to a 15-mL Falcon tube. Add 8 mL of 0.4% water agar supplemented with 100 mg/L cefotaxime. Mix the seed/water agar well.
7. Evenly overlay the mixture on top of the germination medium poured in sundae cup vessels. Allow the seedlings to grow for up to 1 mo, under an 18-h light regime (approximate light intensity of 140 μ mol/m²/s) at 24–28°C. Explants prepared from in vitro germinated seeds will not require the sterilization procedure outlined in **Subheading 3.1.1**.

3.1.3. Leaf Explant Preparation

1. Stack prepared leaves in a Petri plate. Prepare disks using a sterile cork borer (5–8 mm). Avoid leaf margins and mid rib (**Fig. 1A**).
2. Place explants adaxial side up onto the preculture medium (use 15 $\times$ 100-mm Petri plates). Preculture approximately 40 explants per plate. Culture the explants at 25°C under an 18-h light regime (approximate light intensity of 140 μ mol/m²/s) for 24 h.

3.2. Preparation of Agrobacterium Culture (see Note 12)

1. Initiate a 2-mL culture of *A. tumefaciens* in YEP medium supplemented with the appropriate antibiotics. Culture the cells at 28°C with constant shaking. Allow the culture to become saturated. Saturation usually requires 2 to 4 h of culture.

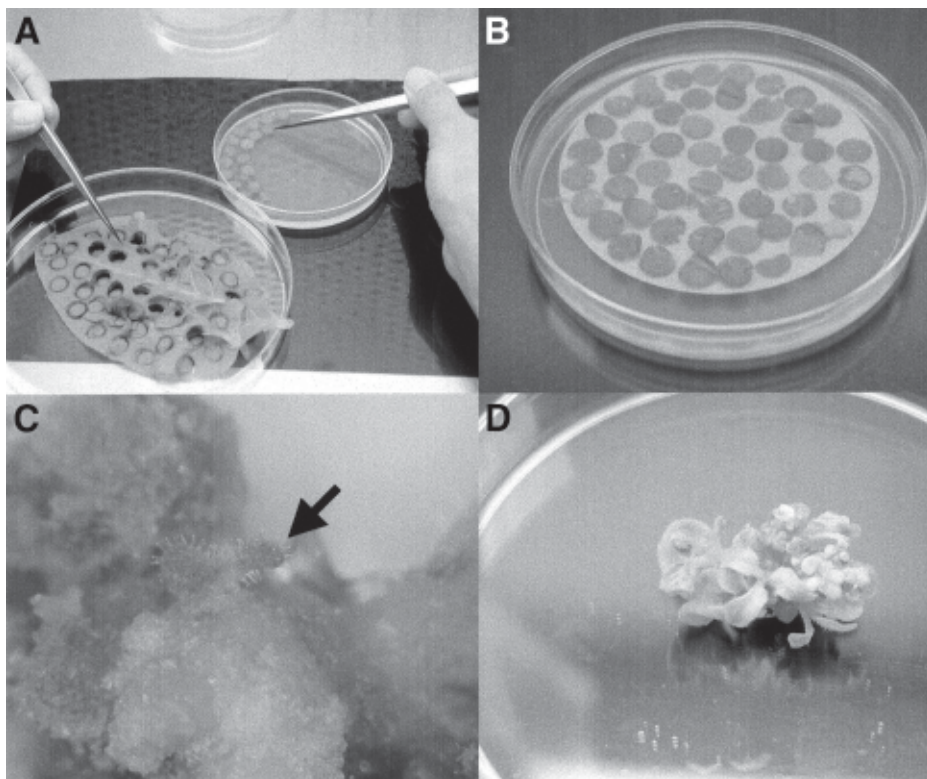


Fig. 1. *N. tabacum* transformation steps. **(A)** Transferring of leaf disks explants to preculture medium. **(B)** Leaf disk explants on cocultivation plates following inoculation. **(C)** Shoot arising (arrow) from a callus cluster. **(D)** Multiple shoot formation.

2. Subculture the 2-mL culture to 50 mL of YEP medium supplemented with the appropriate antibiotics. Culture the cells for an additional 6 to 8 h (28°C with constant shaking).
3. Harvest the cells by centrifugation (3000–4000g).
4. Suspend bacterial pellet to a final OD₆₆₀ of 0.5 to 1.0 in liquid cocultivation medium. Place inoculum on ice until ready for use.

3.3. Plant Transformation (see Note 13)

1. Transfer the prepared *Agrobacterium* inoculum to Petri plates. Inoculate the precultured explants for 30 min.
2. Following the inoculation step, briefly blot explants on sterile filter paper and place, adaxial side up, onto cocultivation medium solidified with 0.8% agar (type A, Sigma), which is overlaid with a single piece of sterile Whatman filter paper. Cocultivation plates should be prepared in 15 × 100-mm Petri plates (see Note 14).



Fig. 2. *N. tobaccum* primary transformant rooted and ready for acclimation step.

3. Cocultivate the explants at 24°C, 18-h light regime for 3 d. Cocultivate approximately 40 explants per plate (**Fig. 1B**).
4. Following the 3-d cocultivation period, transfer the explants to regeneration medium supplemented with the appropriate selection agent. Regeneration medium should be prepared in 15 × 100-mm Petri plates. Culture up to 10 explants per plate and incubate the plates at 28°C under an 18-h light regime. Transfer the explants to fresh regeneration medium every 2 wk.
5. Callus clusters should be apparent forming along the cut parameter of the disk beginning after 2 wk of selection. Multiple shoots can arise from a single callus cluster, and multiple clusters can form from a single disk. Shoots arising from a single cluster will generally be clones. An example of a shoot arising from a callus cluster is shown in **Fig. 1C**; multiple shoot proliferation from a callus cluster is displayed in **Fig. 1D**. Therefore, be sure to track the shoots arising from the same cluster.
6. Excise shoots individually once they reach a length greater than 3 mm and transfer to rooting medium (see **Note 15**) supplemented with the appropriate selection agent. Rooting medium should be poured in a sundae cup vessel or equivalent to allow for sufficient plantlet development.
7. The rooting stage is carried out under the same environmental conditions used during the regeneration step. Roots should be adequately established within 2 wk. A putative *N. tobaccum* primary transformant ready for acclimation to soil is shown in **Fig. 2**.

3.4. Plant Care and Seed Harvest (see Note 16)

1. Carefully remove plantlets with a well-established root system from rooting medium. Wash excess agar off the root system with tap water, transfer the plantlet to a Jiffy-cup, and cover with Metro-Mix 360.
2. Place the Jiffy-cup into a double stacked Magenta vessel and water as needed with 1/2X Hoagland's solution.
3. Place the Magenta vessel into a growth chamber under an 18-h light regime and temperature of 28°C.
4. After 2 to 3 d crack the lid of the stacked Magenta vessel. Once the plantlet is fully acclimated, transfer to the greenhouse by transplanting the Jiffy-cup to a 6-inch pot and cover containing standard greenhouse soil mix.

3.5. Progeny Test and Seed Storage

1. T₁ seed should be collected from fully mature pods. Seeds can be stored in coin envelopes or Eppendorf tubes. Ideal storage temperature for seed is 8°C under low humidity; however, seed viability at room temperature or 4°C remains high for a number of years.
2. To conduct segregation analysis, sterilize harvested T₁ seed, using the sterilization technique described in **Subheading 3.1.2**.
3. After the last washing, transfer the seed to a 15-mL Falcon tube. Add 8 mL of 0.4% water agar supplemented with 100 mg/L cefotaxime. Mix the water agar/seed mixture well by inverting the Falcon tube 5 to 10 times.
4. Overlay agar/seed mixture onto a 100 × 150-mm Petri plate containing approximately 45 mL of germination medium supplemented with the appropriate selection agent. Allow the plates to air-dry with the lids off for approximately 10 min under a laminar flow hood.
5. Place the plates at 28°C under an 18-h light regime. Take segregation data following 2 wk of germination.

4. Notes

1. To select antibiotic compatibility between the *A. tumefaciens* strain and binary plasmid, it is necessary to know the respective drug resistance of the biological material. For example, *A. tumefaciens* strain C58C1 carrying the disarmed Ti-plasmid pPMP90 (13) is resistant to rifampicin (chromosome marker) at a typical use rate of 50 mg/L and gentamicin (Ti-plasmid drug resistance).
2. Store the major and minor salt stock solutions at 4°C.
3. NAA dissolves readily in water. To prepare BAP stock, dissolve powder in 1 M NaOH and bring up in distilled water. pCPA is prepared fresh in 95% ethanol.
4. The antibiotic stocks of carbenicillin and cefotaxime are prepared in water. The stocks may be stored at 4°C for 30 d or frozen in aliquots at -20°C for prolonged storage.
5. Be familiar with the inherent marker genes in the *A. tumefaciens* strains being used and those of the corresponding binary plasmid (see **Note 1**).

6. Pour preculture medium in 15 × 100-mm Petri plates. Plates can be stored for up to 1 mo at 4°C. After chelated iron has been separately prepared, add it directly to the medium prior to autoclaving. Allow the medium to cool in a water bath set at 55°C prior to adding vitamin and growth regulator cocktail solution.
7. Liquid coculture medium can be stored at 4°C for 1 mo. Acetosyringone should be dissolved with a few drops of DMSO first, prior to adding to the vitamin/growth regulator cocktail solution. Pour solid coculture medium in 15 × 100-mm Petri plates. Plates can be stored for up to 1 mo at 4°C.

Cocultivation medium is designed to optimize induction of *A. tumefaciens*, without compromising tobacco cell culture. Hence, reduced salts, low pH, and the inducing agent acetosyringone will provide conditions to maximize for *vir* gene induction with minimal compromise to the *in vitro* culture of the explant. Preventing *A. tumefaciens* cells from engulfing the explants during the cocultivation step is aided by filter paper overlays on the cocultivation plates. This in turn may reduce browning of the explants.

8. The selection medium is poured in 15 × 100-mm Petri plates. Antibiotic stocks for kanamycin and hygromycin should be prepared in water and the concentration based on active ingredients, not merely mass.
9. Salts and sucrose are reduced during the rooting phase. This will tend to increase root induction frequencies.
10. If a series of tobacco transformations will be required, a constant supply of explant material can be established by biweekly seeding flats of tobacco. Culture the stock plants under greenhouse conditions using standard soil mix or Metro-mix 360. The seedlings can be maintained in flats for 2–3 mo.
11. Care must be taken not to soak the leaf tissue in the bleach solution for too long. This will depend on the age of the leaf selected. Generally 8 to 10 min is sufficient.
12. For long-term storage *A. tumefaciens* strains should be kept at –80°C in 25% glycerol. Streak the *A. tumefaciens* strain selected for transformation on a YEP plate supplemented with the appropriate antibiotics and culture at 28°C. It will take about 48 h for the culture to grow sufficiently from a glycerol stock. The plate culture should be used to initiate the liquid inoculum. Starting from a plate will help ensure a clean culture.
13. Transfer the leaf disks from the preculture plate directly to the *A. tumefaciens* inoculum. Be sure the explants are not merely floating on top of the inoculum. Inoculate the explants for 30 min, agitating the bacterium once every 10 min.
14. At the end of the inoculation step, immediately transfer the explants to the cocultivation plates. Following the cocultivation period, transfer the explants to the regeneration medium. Here reduce the explant density per plate to 10. After 1 wk on regeneration plates, check the explants for any visible sign of contamination. If a contaminated explant is observed on a plate, transfer all other explants to a fresh plate and discard the contaminants.
15. Do not try and root more than four shoots per sundae cup. Sweetheart sundae cups *per se* are not labeled “guaranteed sterile,” but if the sleeves are handled like stacks of Petri plates, contamination associated with use of these vessels should

not be a problem. Once roots have been initiated, the plantlets are ready to be established in soil. There is no need to wait for a massive root system to develop.

16. Once a plantlet is transferred out of culture, it should be identified with a unique event number. Keep the plantlet in the stacked Magenta vessel for about 2 to 3 d. After this period crack the upper Magenta so that it sits on the connector with aeration around the corners. If no signs of stress occur on the plantlet after 2 additional days with the upper lid ajar, then the event is ready to be established in the greenhouse. Both *N. benthamiana* and *N. tabaccum* are relatively hardy. Establishing primary transformants out of culture is rarely problematic.

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Generation of Composite Plants Using *Agrobacterium rhizogenes*

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Summary

Limitations in transformation capability can be a significant barrier in making advances in our understanding of gene function through the use of transgenics. To this end we have developed both tissue culture and non-tissue culture-based methodologies for the production of transgenic roots on wild-type shoots (composite plants). Composite plants are generated by inoculating wild-type shoots with *Agrobacterium rhizogenes*, which subsequently induces the formation of transgenic roots. The composite plant system allows for “in root” testing of transgenes in the context of a complete plant and can be analyzed in a variety of gene function analyses and plant-microbe interaction studies. In this chapter we provide a tissue culture-based composite plant generation system for *Arabidopsis* and a non-tissue culture based-method for producing composite plants on a variety of dicotyledonous plant species. Composite plants generated using these methods can be treated like “normal plants,” planted in soil and grown in greenhouses or in growth chambers. These methods have been shown to work efficiently for many different species of plants including several that are recalcitrant to transformation.

Key Words: Composite plants; *Agrobacterium rhizogenes*; transgenics; rockwool; hairy roots.

1. Introduction

One of the essential components of an efficient system for functional gene analysis is the availability of a rapid transformation system for producing transgenic tissue that can be tested. Disarmed *Agrobacterium tumefaciens* has been widely used for producing transgenic plants but has only been adapted for high-throughput transformation of *Arabidopsis* and *Raphanus sativus* (1–3). Numerous plant species have proved to be resistant to the development of high-throughput transformation methods because of either inefficient

transformation by *A. tumefaciens* or recalcitrance of plant regeneration. However, if gene function assays can be done with transgenic tissues without whole plant transformations, methods for transgenic tissue production could prove advantageous provided they can be produced rapidly and inexpensively.

One alternate tool to *A. tumefaciens* that has been used by researchers for the production of transgenic tissues is *A. rhizogenes*. *A. rhizogenes* induces the formation of “hairy roots,” which are capable of rapid shoot-independent growth with minimal to no hormone requirements when grown in tissue culture. *A. rhizogenes*-induced roots are often agravitropic and have an increased number of branching lateral roots. Depending on the strain of *A. rhizogenes* and plant species, *A. rhizogenes*-induced roots often exhibit a “hairy root” phenotype owing to the proliferation and growth of root hairs. *A. rhizogenes*-derived hairy roots have been used extensively in tissue culture to study the interactions between roots and nitrogen-fixing microorganisms (4,5), mycorrhizal fungi (6), and nematodes (7,8). However, the requirements of strict adherence to aseptic technique and the use of costly tissue culture reagents and equipment limit the use of hairy root culture for high-throughput gene analysis.

An alternative to tissue culture-derived hairy root cultures for transgene studies is the composite plant. Composite plants, first described by Hansen et al. in 1989 (9), are produced using the same protocol for the generation of hairy root cultures, except that the newly formed transgenic roots are left attached to inoculated plant shoots rather than excised and cultured separately. The use of composite plants has been documented in several plant interaction studies. Composite plants of *Lotus corniculatus* (9), *Trifolium repens* (4), *Vicia hirsute* (5), and *Arachis hypogaeae* (10) have been used to study gene expression during rhizobia infection and subsequent nitrogen fixation.

In this chapter, we provide two detailed protocols for the production of composite plants. The first protocol describes a non-tissue culture-based system for composite plant production. The second protocol outlines a tissue culture-based system for producing composite plants from those plant taxa that are not amenable to the non-tissue culture-based system. The non-tissue culture-based composite plant system has been developed and tested on a wide range of dicotyledonous plants (11). Depending on the plant species, transgenic roots will begin to appear within 1.5 to 6 wk after inoculation (11). By relying on the natural ability of *A. rhizogenes* to induce root formation, a natural biological selection for transgenic root growth is provided. *A. rhizogenes* will also transfer T-DNAs found on binary constructs introduced and maintained in the bacterium. Transformation efficiencies using the non-tissue culture based-system can reach 100%, producing several transgenic roots per inoculated shoot. The number of independent transgenic roots produced on a shoot can range from 1 to 30 depending on plant species or cultivar used. On average, between

25 and 60% of the roots formed on a shoot are transgenic for the target gene of interest found on the binary vector. Addition of a scorable marker (e.g., green fluorescent protein [GFP]) to the T-DNA provides a means to select transgenic roots visually for analysis and allows for the removal of nontransformed roots. If desired, low doses of selective agents such as kanamycin or glufosinate can raise the efficiencies of transgenic root formation to over 90%. Composite plants can be planted in a variety of growth media (sand, loam, Perlite) and grown in greenhouse or growth chamber conditions similar to wild-type plants. Root tissues can be harvested at any time for analysis. Roots are amenable to DNA, RNA, and protein characterization. Composite plants produced with these methods have been used successfully to study gene function, promoter analysis, RNA interference, and root-microbe interactions (11,12).

Another benefit to using the non-tissue culture-based protocol for composite plant production is that nonsterile tissues can be used to produce roots since a non-sugar-based culture medium is employed. Greenhouse-grown shoots are sufficient for use in most composite plant production procedures. Furthermore, the materials used to produce composite plants are cheap and readily available. Composite plant production uses rock wool (also known as Fibrgro[®]) as a base for holding the *A. rhizogenes* cells and water in close proximity to the cut shoots. The transformation procedure can be conducted under laboratory conditions and requires little skill or plant tissue culture experience.

In certain cases some plants (i.e., *Arabidopsis*), owing to their size, shape, or sensitivity to conditions of composite plant production, may require the use of a tissue culture-based method. To that end we have provided a methodology that uses tissue culture conditions for the rapid production of transgenic plants. The method outlined for *Arabidopsis* composite plant production uses standard tissue culture materials for the formation of composite plants. Approximately 15% of the *Arabidopsis* plants that are treated using the composite plant protocol will become a composite plant. Composite *Arabidopsis* plants are easily distinguished from nontransformed plants by the rapid proliferation of agravitropic and highly branched roots at the base of the hypocotyl. Addition of a scorable marker (e.g., GFP) further aids in the identification of composite *Arabidopsis* plants.

2. Materials

2.1. *Agrobacterium rhizogenes* Strains

We use strain NCPPB 2659 from the National Collection of Plant Pathogenic Bacteria, in York, U.K. (also called K599) because of its wide host range (11). Wild-type *A. rhizogenes* strain K599 is resistant to streptomycin (100 mg/L) and chloramphenicol (5 mg/L) but is susceptible to kanamycin (50 mg/L), spectinomycin (100 mg/L), tetracycline (10 mg/L), and carbenicillin (250 mg/L).

2.2. Binary Plasmids

A. rhizogenes can utilize most binary constructs normally used with *A. tumefaciens*. We currently use a derivative of pBIN19 (13). The bacterial selection of pBIN vectors is the neomycin phosphotransferase II (NPT-II) gene and confers resistance to kanamycin (50 mg/L). Binary plasmids can be introduced into *A. rhizogenes* using electroporation or triparental mating (see Chapter 3).

2.3. Culture Media for *A. rhizogenes*

1. Luria-Bertani (LB) broth: dissolve 10 g/L Bacto-tryptone (Becton Dickinson, cat. no. 211705), 5 g/L yeast extract (Sigma-Aldrich, cat. no. Y-1000), and 10 g/L NaCl (Fisher Scientific, cat. no. S-271) in 1 L of deionized water. Autoclave and then cool to room temperature before adding the appropriate selective agent (depending on the vector and strain being used).
2. LB medium: dissolve 10 g/L Bacto-tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 15 g/L Bacto-agar (Becton Dickinson, cat. no. 214010) in 1 L of deionized water. Autoclave and then cool to 55°C before adding the appropriate selective agent (depending on the vector and strain being used).
3. Kanamycin monosulfate 50 mg/mL stock: place 2.5 g kanamycin monosulfate (PhytoTechnology Laboratories, cat. no. K-378) in a beaker with a magnetic stir bar and 35 mL deionized water. Allow kanamycin to dissolve. Bring final volume to 50 mL with deionized water and filter-sterilize.

2.4. Non-Tissue Culture Composite Plant Protocol

1. ¼ Strength Murashige and Skoog (MS) basal medium: dissolve 1.1 g Murashige and Skoog basal salt mixture (PhytoTechnology Laboratories, cat. no. M-524) in deionized water. Adjust pH to 5.8 using 1 M KOH (Sigma-Aldrich, cat. no. P-1767). Bring volume to 1 L with deionized water. Autoclave for 20 min and store at 4°C.
2. FibrGro® (cat. no. 14-2720, Hummert International, Earth City, MO). Each cube should be cut into 1–1.5-cm cubes. Use a pipet tip to make a hole in each FibrGro® cube.
3. Plastic trays/containers: black flat trays with no holes (cat. no. 11-3050, Hummert International).
4. Clear lids or propagation domes (cat. no. 14-2568, Hummert International).
5. Vacuum desiccator.
6. Sterile Petri dishes (10 × 100 mm).
7. Sterile 250-mL flasks.
8. Sterile 15-mL culture tubes.
9. Soil: Metro Mix 360 (cat. no. 10-0356, Hummert International).

2.5. Arabidopsis Composite Plant Protocol

1. 95% Ethanol.
2. 70% Ethanol, 0.05% Triton X-100: add 262.5 mL of deionized water to 737 mL of 95% ethanol. Add 0.5 mL of Triton X-100 (Sigma-Aldrich, cat. no. T-9284) to solution and mix.

3. $\frac{1}{4}$ Strength Murashige and Skoog (MS) basal medium: dissolve 1.1 g Murashige and Skoog basal salt mixture in deionized water. Adjust pH to 5.8 using 1 M KOH. Take to 1 L with deionized water. Autoclave for 20 min and store at 4°C room.
4. Gamborg's B5 basal salt medium: dissolve 3 g Gamborg's B5 basal salt mixture (PhytoTechnology Laboratories, cat. no. G-768) and 20 g sucrose in deionized water. Adjust pH to 6.1 with 1 M KOH. Add 6 g of Gelrite (PhytoTechnology Laboratories, cat. no. G-469) and take to 1 L with deionized water. Autoclave for 20 min and cool to 55°C before adding selectable agents and/or antibiotics. Pour into sterile Petri plates and store at 4°C.
5. Carbenicillin (500 mg/mL) stock: weigh out 5 g of carbenicillin (PhytoTechnology Laboratories, cat. no. C-346) and dissolve in 10 mL of deionized water. Filter-sterilize (0.2 μ m) and aliquot into 1 mL volumes. This stock will last for 2 to 3 mo when stored at -20°C.
6. Laminar flow hood.
7. Vacuum desiccator.
8. Sterile forceps and scalpel.
9. Sterile filter paper.
10. Sterile Petri dishes (100 $\times$ 15 mm).
11. Sterile 250-mL flasks.
12. Sterile 15-mL culture tubes.
13. Parafilm.
14. Sterile 1.5-mL microcentrifuge tube.

3. Methods

3.1. Non-Tissue Culture Composite Plants

We have developed and tested this protocol for numerous species of plants including tomato, soybean, *Medicago truncatula*, green bean, broccoli, potato, sweet potato, sugar beet, tobacco, okra, and gourd (11). The ease of handling, inexpensive materials needed, and speed for producing transgenic roots on wild-type shoots make this composite plant system valuable to those who are in need of transgenic material for screening purposes (Fig. 1).

3.1.1. Preparation of Explants

Most plants can be used without initial preparation. However, if contamination by endophytic or pathogenic fungi or bacteria becomes a problem, try cleaning the plant seed using chlorine gas prior to planting. Other seed treatments (see Subheading 3.2.1.) may be used if chlorine gas is ineffective.

3.1.1.1. CHLORINE GAS TREATMENT

1. **Caution:** be sure to perform this procedure in a chemical fume hood and not in a laminar flow hood.
2. Place a vacuum desiccator in a fume hood and place seed in an open Petri plate.

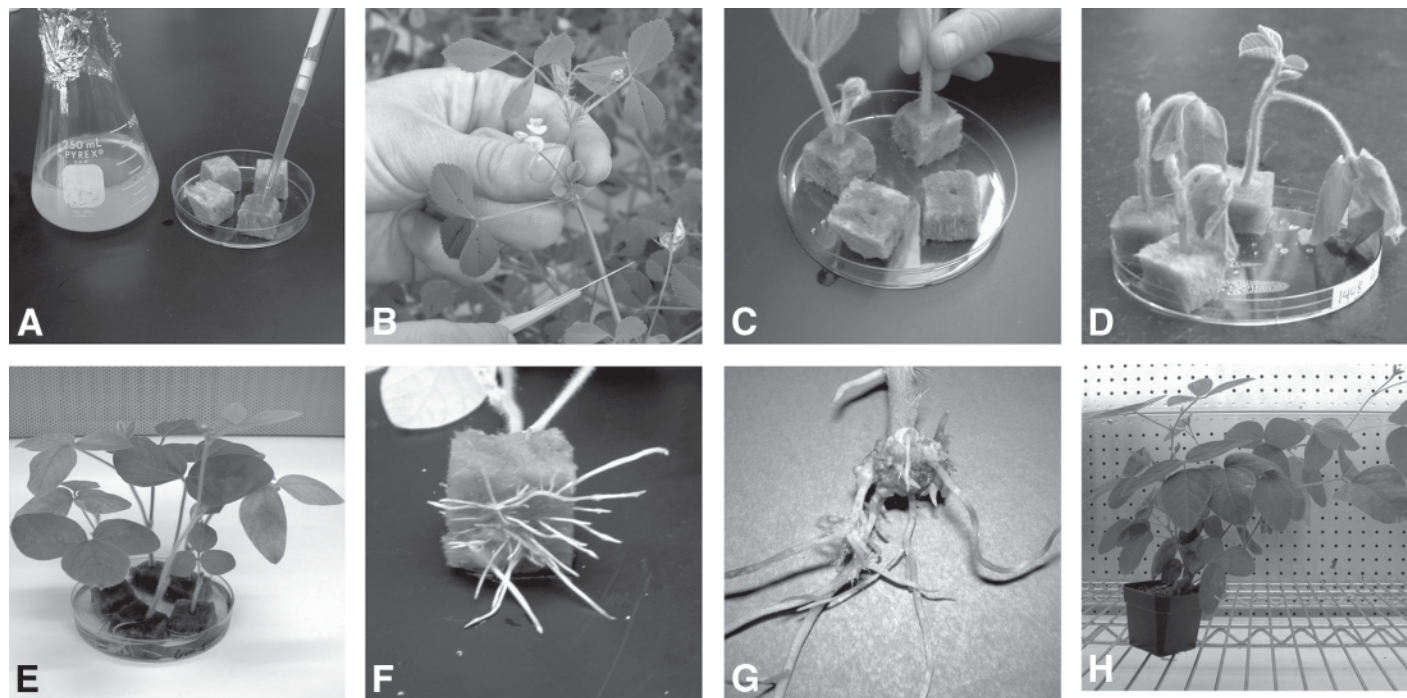


Fig. 1. Non-tissue culture composite plant production. **(A)** Fibragro® cubes are inoculated with *A. rhizogenes* cells from overnight culture pelleted and resuspended to an $OD_{600} = 0.1$ to 0.3 in $\frac{1}{4}$ MS basal medium. **(B)** Shoot material for composite plants is excised from greenhouse-grown plants. **(C)** Excised stem sections are immediately inserted into the inoculated Fibragro® cubes. **(D)** After overnight incubation, plants are allowed to dry to wilting. **(E)** Plants are watered after wilting to restore turgor and continuously watered. **(F)** Roots will emerge from the Fibragro® cubes in a plant taxon-dependent period. **(G)** Removal of Fibragro® cubes reveals the presence of a teratoma from which roots emerge. **(H)** Composite plants can be transplanted to soil and treated as normal plants.

3. In the fume hood add 200 mL of bleach to a 250-mL beaker. Add 2 mL of concentrated HCl to the bleach.
4. Quickly place the beaker and the seed in the vacuum desiccator and close the lid.
5. Pull a vacuum on the desiccator, shut off vacuum, and close valve on desiccator. Let sit overnight.
6. Carefully open up desiccator in the fume hood and quickly cover the seed with the lid of the Petri plate. The seed is now ready for planting (see **Note 1**).

3.1.1.2. PREPARATION OF PLANT MATERIALS

1. Plant seeds in steam-cleaned or autoclaved soil or sand. Any size pot or flat will work provided enough moisture will be retained for proper seed germination.
2. Place pots or flats in the greenhouse and let seeds germinate and plants grow to desired size (see **Notes 2 and 3**).
3. When plants reach desired size they are then ready for inoculation (see **Note 4**).

3.1.2. Preparation of *A. rhizogenes*

1. Streak *A. rhizogenes* from a glycerol stock onto LB medium containing the appropriate antibiotic for vector used. Incubate at 28 to 30°C for 2 d.
2. Transfer one *Agrobacterium* colony from plate into a sterile test tube containing 5 mL of LB broth plus antibiotic selection appropriate for vector used.
3. Place test tube into a 30°C shaker/incubator for 1 d.
4. The quantity of bacterial culture is determined by the number of shoots to be transformed. Each plant will require about 4 mL of final bacterial culture resuspended in $\frac{1}{4}$ strength MS basal medium. Typically the amount of LB broth used is one-third that of $\frac{1}{4}$ strength MS basal medium. This should make the final OD_{600nm} between 0.2 and 0.5. (Example: we decide to transform 25 shoots, thus needing 100 mL of bacterial culture in $\frac{1}{4}$ strength MS basal medium. We will then need to grow the cells in $100/3 = 33$ mL of LB broth.)
5. Transfer 50 μ L of full-grown *Agrobacterium* culture from test tube into a sterile 250-mL flask containing the estimated amount of needed LB broth with antibiotic.
6. Place flask onto a 30°C shaker/incubator overnight.
7. Spin down the cells at 3300g for 10 min. Pour off the supernatant (see **Note 5**).
8. Resuspend the cells with gentle agitation in the appropriate amount of $\frac{1}{4}$ strength MS basal medium to an OD_{600nm} between 0.2 and 0.5.

3.1.3. Inoculation and Coculture

1. Cut FibrGro® (rockwool) sheets to 1–1.5-cm cubes and autoclave.
2. Put three to five cut rockwool cubes into an open Petri dish. Use a 1-mL pipet tip to make a divot (or hole) in the cube.
3. Add 4 mL of resuspended bacteria culture to each cube. Place the open Petri dishes into a plastic tray.
4. Cut shoots off the desired plants. The best shoots to use are those that are young and lack any inflorescences (see **Note 4**). Cut the shoot in the middle of the internode region that is not quite fully expanded.

5. Put one shoot into each cube. Cover the tray with a clear lid and put it in a growth chamber or leave on well-lit benchtop overnight to allow for acclimation. On the second day remove the lid and let cubes dry out. This will cause the tissue to become severely wilted (generally 1–4 d; see **Note 6**).

3.1.4. Explant Recovery

1. If plant selective agents are used, make a watering solution containing the appropriate amount of selective agent in deionized water and use for a one-time application to saturate cubes immediately after the drying step (see **Note 7**). If no selection is to be used, simply saturate cubes with deionized water daily or as needed.
2. Place lids back on trays. Within several hours the plant shoots should recover from wilting.
3. Water plants regularly with deionized water until cubes are saturated (see **Note 8**).
4. If desired, any nontransgenic adventitious roots may be removed. (These are roots that arise in places other than the cut site in the first 2 wk after transformation.) You can visually check whether your tissues appear to be transformed by checking the bottom of the stem for an enlarged and flared structure called a teratoma.
5. Between 1 and 6 wk, roots will start to emerge from the bottom of the stem at the inoculated cut site. If your binary construct contains a scorable marker such as GFP, transgenic roots can be selected by removing all nonfluorescing roots. The plants can be transplanted into soil or other suitable growing medium at this time and treated using conditions suitable for wild-type plants. Roots may have a noticeable agravitropic phenotype (will grow out of the soil).

3.2. Production of Composite Arabidopsis Plants

This protocol details a method for producing composite *Arabidopsis* plants in vitro (**Fig. 2**). We have developed this protocol specifically for use with *A. rhizogenes* strain K599 and *A. thaliana* ecotype Columbia. To aid in identifying transgenic events, we incorporate a constitutively expressed scorable marker (such as GFP) in the T-DNA of the binary plasmid. We are not currently using any selection for our composite plants. Composite plants are readily identified by a proliferation of highly branched roots near the root-hypocotyl interface.

3.2.1. Seed Sterilization

1. In a 1.5-mL microcentrifuge tube, add seeds to a maximum of 100 μ L. (About one tube of seeds can be sterilized and planted on one plate.)
2. In the laminar flow hood, add 1 mL of 70% ethanol, 0.05% Triton X-100 kept at 50°C.
3. Place the tubes on a rotary shaker at 40 to 50 rpm for 20 min at room temperature.
4. After 20 min, remove the ethanol using a sterile pipet.
5. In the laminar flow hood, add 1 mL of 95% ethanol at room temperature.

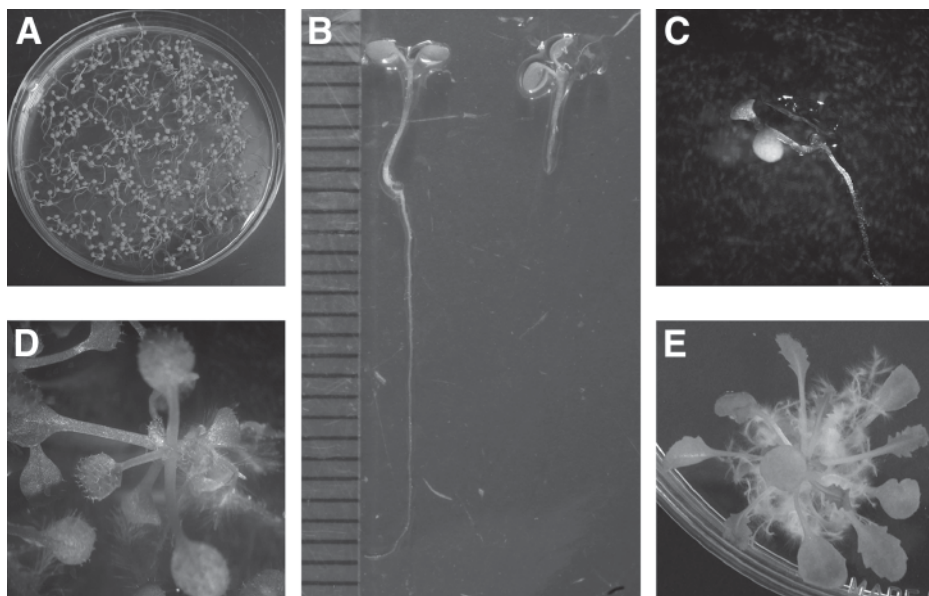


Fig. 2. *Arabidopsis thaliana* composite plant production. **(A)** Wild-type plants at 7 d post germination. **(B)** Plant size of wild-type (on left) and post excision (on right). Scale on left is in millimeters. **(C)** Seven days post inoculation. **(D)** Twenty-one days post inoculation. **(E)** Thirty-five days post inoculation. (Note highly branched root system of composite plant.)

6. Place the tubes on a rotary shaker at 40 to 50 rpm for 20 min at room temperature.
7. Disperse the seeds onto sterile filter paper in an empty 100 × 15-mm dish. Resuspend the seeds as much as possible using the pumping action of the pipetor (see **Note 9**).
8. Allow the filter paper to air-dry in the laminar flow hood.
9. After the alcohol has evaporated, gently pick up the filter papers with sterile forceps and carefully shake the seeds onto Gamborg's B5 basal salt medium plates.
10. Label the plates, wrap them in parafilm to prevent desiccation, and place them in a lighted growth chamber (100 $\mu\text{mol}/\text{m}^2/\text{s}$, 8:16-h light/dark photoperiod, 23°C). Allow the seeds to germinate and grow for 7 d.

3.2.2. Preparation of *A. rhizogenes*

1. Streak *A. rhizogenes* from a glycerol stock onto LB broth plus antibiotic selection appropriate for vector used. Incubate at 28–30°C for 2 d.
2. Transfer one colony from plate into a sterile test tube containing 5 mL of LB broth plus antibiotic selection appropriate for vector used.
3. Place test tube into a 30°C shaker/incubator for 1 d.

4. The day before inoculation, add 10 mL of LB broth plus antibiotics (selection for your binary plasmid) to a flask. Inoculate the liquid media with *A. rhizogenes* from either a single colony grown on LB agar plus antibiotic or from a glycerol stock. Place the cultures on a shaker at 28°C and shake overnight.
5. The day of inoculation, pellet the *A. rhizogenes* cultures at 3300g for 10 min (the pellet should have a slightly pink color). Resuspend the pellet in ¼ MS basal medium to produce a bacterial solution with an OD_{600nm} between 0.2 and 0.3. Typically, we find that a 1:4 dilution is sufficient.

3.2.3. Inoculation and Coculture

1. With a sterile scalpel blade, poke a small hole or slit in the lid of a sterile microcentrifuge tube. This will help in the vacuum infiltration process. Typically, two to three microcentrifuge tubes are needed per germination plate. Plates of 60 × 15 mm can be used instead of microcentrifuge tubes if you are transforming an entire plate of germinated seedlings.
2. Gently pull the plants from the media. Remove most of the root tissue from these plants by placing the roots on the media and, using a scalpel blade, gently slice off the roots by cutting just below the seed coat (root-hypocotyl interface).
3. After the roots are removed, place the explants inside the microcentrifuge tubes (or plates) with care. Fill the tube about one-third to one-half full. Add 1 mL of diluted *A. rhizogenes* solution to each tube. If using plates, add 10 mL of diluted *A. rhizogenes* solution.
4. Place the tubes (or plates) in a vacuum desiccator. Pull a vacuum until the agro/seedling solution shows signs of “bubbling.” Close the vacuum valve and let the tubes sit for 15 min. During the infiltration, prepare coculture plates.
5. Prepare one coculture plate per microcentrifuge tube or three coculture plates per 60 × 15-mm plate. To each sterile plate, add two sterile filter papers and close the lid. Prepare as many plates as needed.
6. After 15 min of infiltration, release the vacuum and remove the tubes (or plates). Open the tubes (or plates) in a laminar flow hood and withdraw the liquid with a sterile pipet. Place the explants on dry filter paper and gently spread them until they are separated (*see Note 10*).
7. Wrap the plates with parafilm to prevent desiccation and place them in a growth chamber (100 µmol/m²/s, 8:16-h light/dark photoperiod, 23°C) for 2–3 d.

3.2.4. Curing and Explant Recovery

1. After coculturing, it is necessary to kill the *A. rhizogenes* by washing the explants with carbenicillin. Prepare 50 mL of ¼ MS basal medium plus carbenicillin (500 mg/L). In a 60 × 15-mm plate, add 20 mL of the ¼ MS plus carbenicillin solution. Add explants to the solution, gently swirl, and let wash for 20 min.
2. After washing, remove the ¼ MS basal medium plus carbenicillin solution with a sterile pipet. Using a sterile forceps or spatula, gently place explants on a sterile filter paper placed on top of Gamborg’s B5 basal salt medium plus carbenicillin (500 mg/L). Wrap the plates with parafilm to prevent desiccation and place them

in a growth chamber (100 $\mu\text{mol}/\text{m}^2/\text{s}$, 8:16-h light/dark photoperiod, 23°C) for 4 to 5 d. This will allow the plantlets to recover and start “standing up,” which will allow for easy transfer.

3.2.5. Hairy Root Selection

1. After 4–5 d, transfer individual plantlets to Gamborg’s B5 basal salt medium plus carbenicillin (500 mg/L) without filter paper to allow for hairy root formation.
2. Two to three weeks after inoculation, hairy roots will be identifiable by the presence of the scorable marker. Additionally, hairy roots will have a highly branched, agravitropic phenotype.
3. After 3 wk, transfer composite plants to Gamborg’s B5 basal salt medium plus carbenicillin (500 mg/L). Place four to five positive plants per plate.
4. Wrap the plates with parafilm to prevent desiccation and place them in a growth chamber (100 $\mu\text{mol}/\text{m}^2/\text{s}$, 8:16-h light/dark photoperiod, 23°C) for an additional 2 to 3 wk.
5. Composite plants can be easily selected if a scorable marker, such as GFP, is used. Alternatively, roots can be tested for transformation simply by excising a small section of roots from test plants and separately cultivating them on plates containing Gamborg’s B5 basal salt medium plus carbenicillin (500 mg/L). Wrap the plates with parafilm to avoid desiccation and place them in a growth chamber (dark, 23°C) for 2 to 3 wk. Wild-type roots will grow slowly, whereas hairy roots will grow rapidly and take over the entire plate.

4. Notes

1. Seed can be checked for cleanliness by germinating a small amount of seed in LB broth, incubating overnight on a 30°C shaker, and checking for growth of bacterial or fungal contaminants.
2. Optimal growing conditions will depend on the species of plant used and will have to be determined. Size of plants can vary, although composite plants can be made from most stem cuttings regardless of age of plant. For making soybean composite plants, we use plants that have two to four trifoliate leaves. For tomato we typically use plants when they have two to four leaves.
3. We have attempted to use plant material grown in the growth chamber but have found that shoots from plants grown in the growth chamber are often susceptible to tip rot.
4. For soybean work we typically cut the stem in the internode region between the meristem and the first fully expanded leaf. For sweet potato we cut tips (meristem plus two to four leaves) of vines and blot to remove any latex that may be exuded before proceeding to the coculture step.
5. The pellet for *A. rhizogenes* strain (K599) should be slightly pink in color.
6. This drying step is necessary for efficient levels of transformation to occur, and the length of time will need to be optimized for each plant species being tested.
7. We have found that only one application of plant selectable agents is needed. If selecting for roots expressing a kanamycin selectable marker (NPT-II), we use

a rate of 10 mg/L of kanamycin. For other selectable markers the concentration of selectable agents will have to be determined. Subsequent watering should be carried out daily using deionized water.

8. Be sure not to overwater the shoots, as this can cause rot. If leaves begin to show signs of yellowing, this often indicates fungal contamination. Fungal contamination can be easily treated using several commercial fungicides (we use a combination of Clearly 3336F and Truban; distributed by Hummert International, cat. nos. 02-0480 and 02-1601, respectively). However, remember that any treatment can affect downstream applications of composite plants.
9. If seeds are slightly larger than the tip hole, the tip may have to be cut slightly with a sterile blade (razor or scalpel) so that the seeds can become suspended inside the pipet tip.
10. It may be necessary to add about 1 mL of $\frac{1}{4}$ strength MS basal medium to the plants to help them spread evenly. If $\frac{1}{4}$ MS is added, then it should be removed after the explants have been spread: tilt the plate and let the liquid drain to the bottom; remove the liquid with a sterile pipet.

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III

CEREAL CROPS

Barley (*Hordeum vulgare* L.)

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Summary

Crop improvement is limited by the availability of valuable traits in sexually compatible species. Access to new characters using genetic engineering would be of great value. Barley has been transformed using microprojectile bombardment and by direct gene transfer to protoplasts, but neither method has been able to produce fertile transformants in large numbers with simple transgene integration characteristics. *Agrobacterium*-mediated transformation was first achieved in 1997, and it has become the method of choice. Using immature embryos of the barley variety Golden Promise as the target organ, the binary vector pWBVec8 containing the intron-interrupted hygromycin resistance gene *hph* as the selectable marker, and selection of transformed cells on hygromycin, the *Agrobacterium* method is efficient, and the transgene insertion characteristics are superior to other methods. However, the procedure is strongly genotype dependent. In this report, we describe a transformation protocol giving details of plant culture, embryo isolation and preparation, vector details, *Agrobacterium* culture, infection methods, subsequent procedures for callus generation and plantlet production, and analysis of transgenic plants.

Key Words: Barley transformation; barley embryos; Golden Promise; *Agrobacterium*; pWBVec8.

1. Introduction

Barley is a major cereal crop, along with wheat, maize, rice, sorghum, oats, and rye, and it is important in the production of food, feed, and beverages; beer in particular. Genetic improvement of barley is limited by its gene pool. Agronomic performance, disease resistance, product quality, and product diversity could be greatly improved with access to genes from sexually incompatible species. Traits already introduced into barley by genetic engineering include immunity to barley yellow dwarf virus (BYDV) (1,2), overexpression of an

enzyme, α -amylase, which is important in the malting and brewing process (3), and production of three foreign proteins including an antibody against the HIV virus (4), a heat-stable β -glucanase to enhance cell wall breakdown during malting (5,6) and a heat-stable xylanase also to assist cell wall breakdown in barley fed to chickens (7). Transformation with green fluorescent protein has also been used as a reporter for gene promoter function (8) and to indicate transformation (9).

Barley was first transformed using DNA-coated microprojectiles in 1994 (10) and subsequently by direct gene transfer into protoplasts in 1995 (11) and by *Agrobacterium* mediation in 1997 (12). The ability to produce stably transformed fertile barley plants varies considerably between transformation methods. Although gene transfer into protoplasts is easily accomplished, regeneration of large numbers of fertile plants is difficult. This is probably largely a problem with protoplast regenerability rather than gene insertion. Bombardment of cultured tissue has been more successful. All the major cereals have been transformed in this way. However, the number of gene insertion sites and the number of gene copies inserted is usually large, genes are often not intact, and segregation of the inserted genes is complex. Transformed plantlets of barley can be produced in large numbers, but transformants with single insertion sites containing low gene copy numbers are low. Transformation of barley with *Agrobacterium* has proved to be superior to other methods. Not only can transformants be produced in high numbers, but also the gene insertion characteristics are superior. Integration of the transgene into the genome is (to an extent) defined, and transgene copy number is low, with single insertion sites containing one to several gene copies being common (about 50%). No transgene silencing has been reported with *Agrobacterium*, whereas it can be a problem with bombardment (13). An additional advantage of using *Agrobacterium* is the ability to insert independently the gene of interest and the selectable marker gene and subsequently segregate away the marker gene, thus producing selectable marker-free lines (14). However, although the *Agrobacterium* method is highly efficient using the barley cultivar Golden Promise, first used by Wan and Lemaux (10), the efficiency is mainly owing to the regenerability of the cultivar. Thus the method is highly genotype dependent, and other elite cultivars of barley have been transformed (e.g., refs. 2 and 15), only with considerably less efficiency than Golden Promise (15).

This article describes a method for *Agrobacterium*-mediated transformation of barley developed in this laboratory using the *hph* gene. Two modifications have been made since the original report (12). The particle bombardment procedure has been deleted (see also ref. 16), and the vector has been improved. In brief, immature barley embryos of the barley cultivar Golden Promise are cocultivated with *Agrobacterium* containing the plasmid pWBVec8 (17).

This is followed by callus induction, selection of transformed cells on hygromycin, and then plantlet regeneration. Using Golden Promise as the recipient cultivar, our transformation efficiencies average about 10% (range 6–50%) (i.e., 10 independently transformed fertile plants from each 100 embryos cocultivated with *Agrobacterium*). Efficiency can be affected by the transgene (15) as well as other factors, many as yet not identified. About 50% of the transformants give 1:3 segregation of the transgenes in the T₂ generation, indicating single insertions (but not necessarily single genes) into the genome (see **Note 1** concerning gene copy number).

2. Materials

2.1. Plant Material

1. Barley cultivar Golden Promise seed: Golden Promise is a husked spring barley bred in Europe and grown to a small extent commercially. It was first used by Wan and Lemaux (10) to transform barley by particle bombardment because of its excellent ability to form embryogenic callus and ultimately regenerated plantlets. We obtained seed from the Lemaux laboratory, and it can be obtained from us.
2. Australian elite cultivars Schooner, Sloop and Chebec seeds: These are also husked spring barleys bred in Australia and grown commercially. Seed can be obtained from the authors.

2.2. Vector

The vector most commonly used is pWBVec8 (17,18; Fig. 1) This vector features (1) the hygromycin phosphotransferase *gene* (*hph*), which confers resistance to hygromycin B as the selectable marker (see **Note 2**), (2) a number of restriction sites that permit the insertion of target genes, (3) an *E. coli* origin of replication that allows for high-copy-number replication of the plasmid in *E. coli* and hence high yields of plasmid DNA preparations, and (4) an intron in the *hph* gene that prevents expression in bacteria, rendering it susceptible to hygromycin and thus allowing the control of bacterial overgrowth in tissue culture with hygromycin present. Hygromycin is therefore both selective for transformed plant cells and lethal to *Agrobacterium*.

2.3. Stock Solutions

For the solutions described in **items 1–3**, all reagents should be added one at a time, allowing each to dissolve before adding the next. This prevents a precipitate from forming. All the following solutions should be kept at 4°C.

1. MS macronutrients (10X stock): 16.5 g/L NH₄NO₃, 19.0 g/L KNO₃, 4.4 g/L CaCl₂·2H₂O, 3.7 g/L MgSO₄·7H₂O, 1.7 g/L KH₂PO₄.
2. FHG-II micronutrients (100X stock): 0.62 g/L H₃BO₃, 1.69 g/L MnSO₄·H₂O (or 2.23 g/L MnSO₄·4H₂O), 0.86 g/L ZnSO₄·7H₂O, 0.083 g/L KI, 0.025 g/L

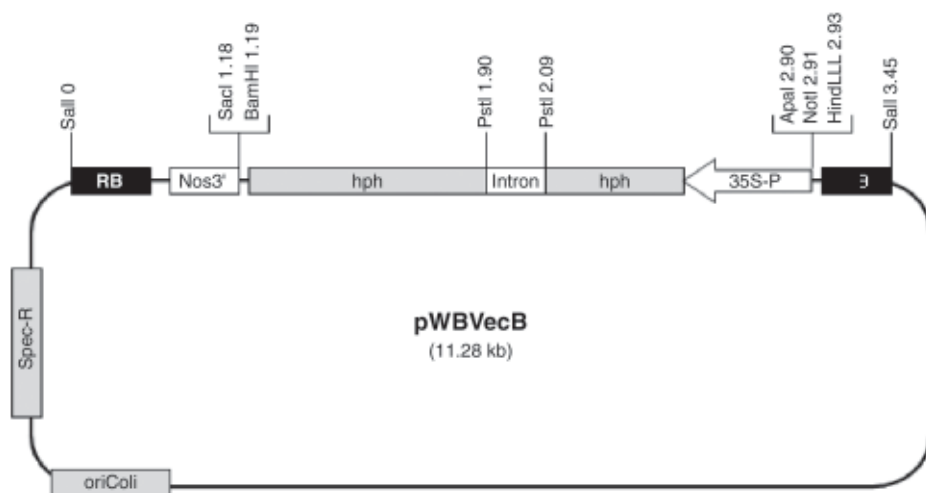


Fig. 1. A simplified map of pWBVec8. Only a few of the restriction sites are shown, including the unique cloning sites *ApaI*, *NotI*, and *HindIII*. The relative positions of these restrictions sites in the plasmid are also given. Spec-R, spectinomycin resistance gene; oriColi, the *E. coli* origin of replication; RB, T-DNA right border sequence; LB, T-DNA left border sequence; 35S-P, the cauliflower mosaic virus 35S promoter; hph, hygromycin resistance gene coding sequence; Intron, a modified version of the castor bean catalase intron 1; Nos3', the transcriptional termination sequence of the *Agrobacterium* nopaline synthase gene.

$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.0025 g/L $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (or 0.0016 g/L CuSO_4 ; or 1 mL of stock 0.0025 g/mL), 0.0025 g/L $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (or 1 mL of stock containing 0.025 g/mL).

3. FHG-I macronutrients (10X stock): 1.65 g/L NH_4NO_3 , 19.0 g/L KNO_3 , 4.4 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 3.7 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.7 g/L KH_2PO_4 .
4. Iron (200X stock): 1.08 mL of FeCl_3 (purchased as a 60% solution) in 200 mL water. This solution is light sensitive and must be stored in the dark at 4°C.
5. EDTA (20 mM stock): dissolve 7.44 g $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$ in 1 L water.
6. Thiamine (1 mg/mL stock): dissolve 50 mg thiamine-HCl in 50 mL water.
7. Dicamba (1 mg/mL stock): dissolve 100 mg dicamba in 3 drops 1 M NaOH and make up to 100 mL in H_2O .
8. Biotin (0.1 mg/mL stock): dissolve 1 mg biotin in 10 mL water.
9. Rifampicin (25 mg/mL stock): dissolve rifampicin in water.
10. Spectinomycin (50 mg/mL stock): dissolve spectinomycin in water.

2.4. Working Solutions

1. BCI-DM Callus Induction Medium: 30 g/L maltose, 5 mL/L EDTA (20 mM stock), 5 mL/L iron (200X stock), 100 mL/L MS macronutrients (10X stock), 10 mL FHG-II

- micronutrients (100X stock), 1 mL thiamine-HCl (1 mg/mL), 250 mg/L myo-inositol, 1 g/L casein hydrolysate, 2.5 mL/L dicamba (1 mg/mL), 0.69 g/L proline (see **Note 3**).
2. FHG shoot induction medium: 100 mL/L FHG-I macronutrients (10X stock), 10 mL/L FHG-II micronutrients (100X stock), 1 mL/L thiamine-HCl (1 mg/mL), 5 mL/L iron (200X stock), 5 mL/L EDTA (20 mM stock), 1 mL/L 6-benzylaminopurine (1 mg/mL), 100 mg/L myo-inositol, 730 mg/L L-glutamine, and 62 g/L maltose. Adjust pH to 5.9 and then add 3.5 g/L of Phytigel. Autoclave and then add 20 mg/L hygromycin and 100 mg/L timentin for plates.
 3. BCI root induction medium: this is the same as BCI-DM medium except that it contains no dicamba. Adjust pH to 5.9 and then add 3.5 g of Phytigel to 1 L. Autoclave and then add 20 mg/L hygromycin and 100 mg/L timentin.
 4. MG/L medium: 5 g/L mannitol, 1.0 g/L L-glutamic acid, 0.25 g/L KH_2PO_4 , 0.1 g/L NaCl, 0.1 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 $\mu\text{L/L}$ biotin (0.1 mg/mL stock), 5 g/L tryptone, 2.5 g/L yeast extract. Adjust pH to 7.0 and autoclave. For MG/L plates, add 1.5% agar.
 5. LB medium: LB medium contains 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl. The pH is adjusted to 7.5, 15 g/L of agar is added, and the mixture is autoclaved and poured into Petri dishes.
 6. Leaf assay medium: MS salt mixture, pH 5.7, 0.5 mg/L 6-benzylaminopurine, 100 mg/L hygromycin, and 8 g/L agarose or agar.
 7. Bead lysate solution 1 (BL1): 4.78 g KCl, 3.2 mL 5 M KOH, 1.6 mL 0.5 M EDTA and water to a total of 80 mL.
 8. Bead lysate solution 2 (BL2): 16 mL 1 M Tris-HCl (pH 9.5), 4.78 g KCl, 3.2 mL 5 M HCl, 1.6 mL 0.5 M EDTA, and water to a total of 240 mL.

3. Methods

3.1. Plant Culture

Golden Promise barley plants are grown in a growth cabinet at 12°C constant temperature, with a 16/8-h (d/night) light regime. Australian elite cultivars (e.g., Schooner, Sloop, and Chebec) are grown at 18/13 (d/night) with the same light regime. Light is provided by four 100-W incandescent globes and six 400-W metal halide globes providing 400 $\mu\text{mol/m}^2/\text{s}$ at (barley) head height. Plants are grown in medium consisting of compost/perlite (80/20) in 15-cm plastic pots. Several pots are sown each week to ensure an ongoing supply of seed at a suitable stage of development for transformation.

3.2. *Agrobacterium* Preparation

1. Streak the *Agrobacterium* strain carrying the vector (see **Note 4**) onto LB plates containing rifampicin (25 mg/L) and spectinomycin (50 mg/L). Grow the *Agrobacterium* at 27°C for 2 to 3 d.
2. Take a single colony of the *Agrobacterium* culture and inoculate 5 mL of MG/L medium containing rifampicin (25 mg/L) and spectinomycin (50 mg/L).

3. Shake vigorously at 27°C for 24 to 40 h to produce a well-grown solution (OD_{600} about 0.5). This culture (culture A) is used for preparing stocks for both archiving and for transformation.

3.2.1. Prepare Glycerol Stocks for Archiving

1. Take 100 μ L of culture A, spread it onto an LB plate containing rifampicin (25 mg/L) and spectinomycin (50 mg/L), and grow for 2 to 3 d at 27°C.
2. Divide the plate in half by drawing a line across the bottom of the plate. Using a flamed loop, transfer as much as possible of the *Agrobacterium* from one-half of the plate into 1 mL of sterile 30% glycerol/LB (375 μ L of sterile 80% glycerol plus 625 μ L of LB) to make a thick solution.
3. Vortex the culture, and then leave it on the bench for a few hours, mixing every 0.5 h until well mixed. Snap-freeze the culture in liquid nitrogen and store at -80°C .

3.2.2. Prepare Glycerol Stocks for Transformation

1. Add 200 μ L of culture A to 200 μ L of 30% aqueous glycerol (previously sterilized) in sterile Eppendorf tubes.
2. Vortex, and then leave on the bench for a few hours, mixing every 0.5 h until well mixed. Store at -80°C . Prepare 10 to 20 tubes per construct.

3.3. Transformation Procedure

3.3.1. Seed Collection, Plate Preparation, and Preparation of the *Agrobacterium* Culture (Day 1)

1. Harvest seed about 14 d after flowering. Pick about 10 barley heads of the correct size (see **Note 5**).
2. Remove the awns from the seeds and remove the seed from the heads.
3. Place the seeds in a 50-mL Falcon tube (no more than half full), rinse with 70% ethanol, shaking for 1 min, and then discard the ethanol.
4. Immediately add a bleach solution containing 1% hypochlorite and a drop of Tween-20 per 200 mL. Add the bleach solution to the seeds and mix on a shaker for 20 min.
5. In a laminar airflow hood, pour off the bleach solution and wash the seeds with sterile water, shaking for 1 min (repeat six times). Drain off as much water as possible and place at 4°C overnight.
6. Initiate an *Agrobacterium* culture by adding 100 μ L of thawed glycerol stock (see **Subheading 3.2.2.** above) to 5 mL of MG/L medium and place on a shaker at 27°C overnight. The shaker is kept in the cold room to ensure maintenance of temperature at 27°C .
7. Prepare BCI-DM plates on Day 1 or the day before (see **Note 6**).

3.3.2. Embryo Isolation, Excision of Embryonic Axis, and Infection with *Agrobacterium* (Day 2)

This and all subsequent procedures must be undertaken in a laminar flow hood.

1. Using a scalpel and microforceps, place some seeds in a sterile Petri dish on a microscope platform. Locate the position of the embryo (beneath the hilum), and position seed such that the hilum is down (i.e., crease up).
2. Cut the tip off the seed to open up the embryo cavity, taking care to avoid damage to the embryo. Alternatively, to avoid embryo damage, open the end of the seed by gently teasing with a scalpel. Holding the seed with forceps, press down gently on the middle of the seed just behind the embryo with the side of the scalpel blade. The embryo should pop out easily.
3. Support the isolated embryo with the side of the tips of the forceps (gripping with the forceps will damage the embryo), insert the scalpel blade into the groove between the scutellum and the embryo axis, and carefully excise the axis (see **Note 7**).
4. Discard the seed and place the embryo (minus the axis) cut side up (i.e., scutellum down) in the center of a BCI-DM plate (antibiotic free; see **Note 8**). Put approximately 25 embryos on each plate as close together as possible in a compact array without overlapping each other.
5. Take approximately 20 μL of the *Agrobacterium* culture, and place several drops onto the embryos on the (antibiotic-free) BCI-DM plate, ensuring that all embryos come in contact with the solution (see **Note 9**).
6. Flip the embryos over so that the cut side is down. One by one drag the embryos across the surface of the medium halfway to the edge of plate; then turn the embryos over and drag them the remainder of the way to the edge (see **Note 10**). This removes excess *Agrobacterium* and reduces overgrowth.
7. Transfer the embryos to fresh (antibiotic-free) BCI-DM plates (cut side up) and arrange at evenly spaced intervals (about 25 per plate; see **Note 11**).
8. Seal plates with Micropore tape. Place in a dark cabinet or lightproof box at 24°C for 3 d (see **Note 12**).

3.3.3. Embryo/Callus Culture and Plantlet Production (Day 5 and Beyond)

1. After *Agrobacterium* cocultivation, transfer embryos cut side up onto BCI-DM plates containing timentin and hygromycin. From here on, all BCI-DM plates contain timentin and hygromycin.
2. Embryos/calli are transferred onto fresh BCI-DM medium every 2 wk (for a total of 6 wk), keeping plates in the dark at 24°C (see **Note 13**). Embryos should then be showing good callus formation and a substantial increase in size.
3. Transfer the calli onto FHG (shoot induction medium) and move into the light at 24°C. Calli are transferred onto fresh FHG plates every 2 wk, keeping plates in the light at 24°C (see **Note 13**).

4. Shoots begin to appear from 4 to 6 wk on FHG. Continue transferring both callus and small shoots for up to 16 wk, regularly removing dead (brown) callus from cultures from 6 wk onward.
5. When shoots outgrow the Petri dish (starting to bend at the tips), transfer them into pots containing BCI (root induction medium; see **Note 14**). Some gentle scraping away of callus at the base of the plantlet is required to prevent callus growth that could hinder root formation.
6. Roots begin appearing 1 wk after transfer to BCI.
7. When several strong white roots are visible (or secondary roots appear), transfer to Jiffy (peat) pots. To do this, wet the pots, add soil (80% compost, 20% perlite), and wet lightly again.
8. Gently tease plantlets out of the BCI medium and wash off any traces of gel from the roots before planting in soil.
9. Place plants in a shaded misting bench (in a glasshouse set at 17/9°C [day/night]) for 10–16 d, or as long as it takes either for roots to emerge from the sides of the Jiffy pots or for new leaf growth to occur.
10. Plantlets are then transferred into soil in plastic pots (15 cm diameter) in a glasshouse maintained at 17/9°C (day/night; see **Note 15**).
11. From transplanting seedlings to grain harvest takes about 7 mo. Seed set is comparable to untransformed Golden Promise in most cases.
12. Seedlings are fertilized with slow-release fertilizer pellets mixed into the soil. Golden Promise is relatively susceptible to disease, and seedlings must be sprayed against powdery mildew (and perhaps other fungi).

3.4. Detection of the Transgenes Using PCR Analysis

Detection of both the *hph* gene and the gene(s) of interest in callus and plantlet/seedling tissues (leaf or root tissues) can be done by polymerase chain reaction (PCR) (**14**). A rapid method for DNA extraction from barley tissues that works for both leaf and root tissues has been described in detail previously (**14**).

1. Excise about 10 mm² of young leaf tip tissue or three to four tips of young roots about 4 mm long and place in 0.5-mL Eppendorf tubes with 50 µL of BL1 solution.
2. Add three glass balls (1.5–2.0 mm diameter, BDH, cat. no. 332134Y), cap the tubes, and place up to 14 tubes in a small plastic screw-cap jar attached to a Vortex mixer. Run the mixer on high for 2 min (see **Note 16**).
3. Neutralize the lysate by adding 150 µL of BL2 solution (final pH about 7.6), vortex, and then centrifuge.
4. The DNA extracts are now ready for PCR analysis.
5. The *hph* gene can be reliably detected by PCR using a primer pair amplifying a 917-bp sequence (5'-ACTCACC GCGACGTCTGTC-3' and 5'-GCGCGTCTGCTGCTCCAT-3') (**14**).
6. Perform PCR in 10 µL containing 1.0 µL of crude tissue lysate, 9.5 µL of a solution giving final concentrations of 10 mM Tris-HCl, pH 8.2, 3.5 mM MgCl₂,

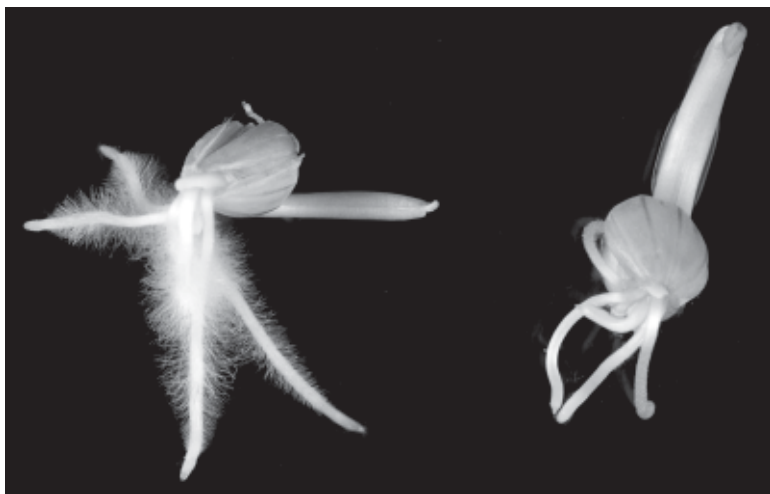


Fig. 2. Root hair growth is abundant on barley seedlings grown in water (left) but is strongly inhibited on seedlings grown in 100 mg/L hygromycin (right). Further details are given in **Subheading 3.5.1**.

200 μM of each dNTP, 0.5 μM of each primer, and 0.01 U/ μL of Taq DNA polymerase.

7. Genes of interest can be detected simultaneously or subsequently using appropriate primers.

3.5. Progeny Screening Using Hygromycin

The *hph* gene can also be detected in leaves and seeds by determining susceptibility/resistance to hygromycin.

3.5.1. Root Assay

Hygromycin is a potent inhibitor of root hair production in germinating barley seeds (J.V. Jacobsen, unpublished; **Fig. 2**), and this can be used as an efficient unambiguous screen for the presence of the *hph* gene and the determination of segregation ratios.

1. Place 20–25 seeds evenly spaced, crease down, and all pointing in one direction (to evenly space root clusters when they appear), on agar (0.7 $\mu\text{g}/100\text{ mL}$) plates containing 100 mg/L hygromycin (see **Note 17**).
2. Seal the dishes with parafilm, wrap in aluminium foil, and incubate at 4°C for 7 d to eliminate dormancy and produce uniform germination.
3. Remove the foil and put the plates in a growth room at 20°C under light (about 100 $\mu\text{mol}/\text{m}^2/\text{s}$) for 24 h. Light is provided by Sylvania Luxline plus F36W/860 Daylight De Luxe fluorescent tubes (see **Note 18**).

4. If the *hph* gene is present, roots have abundant root hairs; when the gene is absent, root growth is slightly retarded and there are few, if any, root hairs. When root hairs are abundant, they penetrate the agar and anchor the seedling, but when root hairs are absent, this does not occur. Consequently, nulls can be easily separated from transgene-positive seeds by turning the plate upside down when nulls fall out.

3.5.2. Leaf Assay

Barley leaves are relatively sensitive to hygromycin. Leaf assay can therefore be used to differentiate transgenic plants from nontransgenic ones.

1. Excise two or more healthy green leaf tips about 1–2 cm long from each plant and immediately place into leaf assay medium in Petri dishes with the cut ends embedded to allow good contact with the medium (see **Note 19**).
2. Place the Petri dish in a 24°C growth room with a 16/8-h light/dark regime.
3. Leaves of nontransgenic plants should be bleached after about 7 d, but those of transgenic plants should remain green.

4. Notes

1. About 80% of transformed (containing the *hph* gene) plants have the gene of interest; 50% of these have single copies of the gene of interest (single insert), 30% have two copies, and the rest have three or more.
2. The choice of selectable markers to produce transgenic barley at high efficiency is limited. Like other cereals such as rice, barley tissue is relatively insensitive to the antibiotic kanamycin, and therefore the *nptII* gene, which confers kanamycin resistance and is widely used in dicot transformation, has not been very useful for barley transformation. Two selectable marker gene systems have so far been successfully used in barley transformation, the *bar* gene and the *hph* gene. A major drawback with the *bar* gene is that the selection is not effective with large callus pieces, but an advantage is that the gene can be readily followed in subsequent generations with foliar spraying. The *hph* gene, encoding hygromycin phosphotransferase, which phosphorylates hygromycin B and detoxifies it, is preferable.
3. When making 1 L of BCI-DM medium, put maltose, EDTA, and iron in 250 mL and all other ingredients in 750 mL. Adjust pH of both solutions to 5.9, and then add 3.5 g/L Phytigel to the 750 mL, autoclave, and combine the two after autoclaving. Days 2–4 require antibiotic-free plates and Day 5 onward require plates containing 100 mg/L timentin and 50 mg/L hygromycin. The ingredients EDTA, iron, MS macronutrients, and FHG-II can be substituted with 4.3 g of the MS salt mixture available from Gibco (cat. no. 11117-074).
4. Vector construct can be introduced into *Agrobacterium* strain AGL1 or AGL0 by triparental mating or electroporation. Although both strains give high frequencies of transformation, AGL1 is preferable because the integrity of plasmids is probably better maintained than in AGL0.

5. Seed collection can be performed on Day 1 or on the morning of embryo isolation (Day 2). The plant material must be healthy. Use heads from primary tillers at the required embryo size. The seeds should be green and disease free. We have found barley embryo size to be a very important factor in transformation and regeneration performance. The scutellum should be about 2 mm in diameter, with the embryos not appearing watery and translucent (too young), or firm and creamy yellow (too old).
6. Media should not be more than 1 wk old when transferring. However, plates stored at 4°C could be used after a longer period. Note also that both hygromycin and timentin are heat sensitive, especially timentin. Therefore it is necessary to cool the medium down before adding these components.
7. The embryos are fragile and are easily bruised and cut. We have found that the best results come from embryos with minimal damage. This is a difficult operation and might require several days of practice before satisfactory embryos are consistently obtained. A sharp scalpel facilitates this process, so regular replacement of the blade is necessary. Curved nosed microforceps are particularly useful for holding the embryo in place while excising the axis.
8. During *Agrobacterium* infection (i.e., Days 2–4), timentin and hygromycin are not present, enabling *Agrobacterium* to grow.
9. Only the minimum amount of *Agrobacterium* culture necessary to coat the embryos should be used. This reduces overgrowth and increases callus growth. Also, we have found that inclusion of acetosyringone in the cocultivation medium, which is essential for rice transformation, does not improve the efficiency of Golden Promise barley transformation.
10. Discard any embryos that are damaged in the dragging process.
11. Do not drag the embryos across the surface of the fresh BCI-DM plate *at all* when transferring—this creates bacterial colonies next to the embryo, which will quickly engulf it. At Day 4, if callus is overgrown with bacteria, transfer to fresh plates. However, it is better to use less *Agrobacterium* on Day 2 than to transfer on Day 4.
12. Callus must not be allowed to become wet from condensation. To prevent this, temperature must be constant at 24°C. If condensation appears, plates must be opened and allowed to dry in a laminar flow hood.
13. If fungal or bacterial contamination occurs during the transfers, infection can be eliminated by including PPM (Plant Preservative Mixture, Plant Cell Technology, Washington, DC) in the medium (Craig Jackson, CSIRO, unpublished data). Callus growth does not appear to be affected.
14. BCI pots are plastic screw-cap jars 65 mm in diameter by 75 mm high. They can be reused, but they must be washed and rinsed thoroughly, autoclaved, and then dried before further use.
15. Retain all plantlets because a number of independent transformants can be recovered from a single callus line (12).
16. In order to shake the tubes vigorously, we mounted a plastic “urine specimen” jar (about 50 × 42 mm diameter) on the top of a vortex mixer. We then put 8–14 of

the 0.5-mL Eppendorf tubes (tightly capped) in the jar on their sides and vortexed for about 2 min at maximum noise. After this, the extracts should be slightly green. If not, repeat the vortexing process.

17. Use 9-cm-wide Petri dishes containing 70 mL of 0.7% agar.
18. We don't know whether the spectral properties of these tubes are critical, but they may be.
19. Fungal or bacterial contamination does not usually cause problems for the assay because the medium is basically free of organic nutrients.

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Maize (*Zea mays* L.)

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Summary

Agrobacterium tumefaciens-mediated transformation is an effective method for introducing genes into maize. In this chapter, we describe a detailed protocol for genetic transformation of the maize genotype Hi II. Our starting plant material is immature embryos cocultivated with an *Agrobacterium* strain carrying a standard binary vector. In addition to step-by-step laboratory transformation procedures, we include extensive details in growing donor plants and caring for transgenic plants in the greenhouse.

Key Words: *Zea mays*; maize; genetic transformation; *Agrobacterium*-mediated; *Agrobacterium tumefaciens*; standard binary vector.

1. Introduction

Transgenic maize is among one of the first biotechnology crops globally commercialized. A number of gene delivery systems such as the biolistic gun, electroporation, silicon carbide whiskers, and *Agrobacterium tumefaciens* infection can be used for maize transformation (1). One of the greatest advantages of using the *Agrobacterium*-mediated transformation method is its ability to generate large numbers of maize events with single or relatively low transgene copy numbers (2–4). In general, simple transgene insertion is preferred because these transgenic plants are less prone to multisequence-induced gene silencing (5) and have been shown to maintain higher and more stable transgene expression over generations (4). However, one of the major challenges in implementing this method is that it involves balancing interactions between two living organisms, the plant and the bacterium, to achieve success. Cells of one maize genotype or tissue type may be transformable using the biolistic gun but not readily amenable to transformation using the *Agrobacterium* method if they are not susceptible to infection by this biological

delivery agent. For example, we have achieved stably transformed plants from immature embryo scutellum cells of the inbred line Oh43 using the biolistic gun but have been unsuccessful in achieving transient or stable transformation of the same genotype using the *Agrobacterium* method (B. Frame, unpublished data).

To date, the most widely targeted explant for *Agrobacterium*-mediated stable transformation of maize is the immature zygotic embryo (2,3,6,7). Efficient, stable transformation has also been achieved using type II callus for infection (8). Transgenic R0 plants were regenerated after infecting proliferating shoot apical meristem cultures (9) or shoot apical meristems themselves (10) with *A. tumefaciens*, but transmission of the transgene to progeny plants was not confirmed.

In this chapter, we describe the *Agrobacterium*-mediated maize transformation protocol used routinely in our laboratory (6) to transform immature embryos of the maize Hi II genotype. *A. tumefaciens* strain EHA101 harboring a standard binary vector is used to infect the embryos and deliver transgenes into plant cells. Type II callus cultures induced from the infected embryos are selected on bialaphos-containing media, and putative transgenic plants are regenerated from bialaphos-resistant, somatic embryogenic callus. The process (from nontransgenic donor embryos to transgenic maize seed) requires approximately 200 d (Fig. 1). The average transformation efficiency of this system is 5% (defined as 5 bialaphos-resistant callus lines recovered from 100 infected immature embryos) and ranges from 1 to 20% depending on the gene of interest.

2. Materials

2.1. *Agrobacterium tumefaciens* Strain and Vector

A. tumefaciens strain EHA101 (11) carrying the 9186-bp standard binary vector pTF101.1 (12) derived from pPZP is used (13). This vector contains the *bar* gene selectable marker cassette, which confers resistance to phosphinothrycin, the active ingredient in bialaphos (14). The *bar* gene is driven by the double 35S CaMV promoter (15) and is flanked on the 5' end by a tobacco etch virus (TEV) translational enhancer (16) and on the 3' end by the soybean vegetative storage protein terminator (17). pTF101.1 also contains a multiple cloning site (MCS) for insertion of a gene of interest (GOI) (see Note 1).

2.2. Plant Material

1. Maize Hi II F1 seeds (see Note 2): ears of the maize Hi II genotype (A188 × B73 origin [18]) harvested from greenhouse-grown embryo donor plants 9–13 d after pollination. Immature zygotic embryos (1.2–2.0 mm) aseptically dissected from these ears are targeted for *Agrobacterium*-mediated transformation (6,19) without preculture.
2. Maize B73 seeds (see Note 2): as the pollen donor plant.

<u>Step</u>	<u>Action</u>	<u>Day of Action</u>	<u>Efficiency Cascade</u>
1	Infect immature zygotic embryos with <i>A. tumefaciens</i> and begin co-cultivation	Day 1	100 immature embryos 
2	Transfer all embryos from co-cultivation to resting medium	Day 3	
3	Transfer all embryos to selection medium containing 1.5 mg/L bialaphos	Day 10	
4	Transfer all embryos to selection medium containing 3 mg/L bialaphos	Day 24	
5	Transfer all embryos to selection medium containing 3 mg/L bialaphos	Day 38	
6	Pick independent transgenic events (callus)	Day 52	5 putative transgenic events 
7	Regenerate putative transgenic events (dark)	Day 68	
8	Germinate transgenic events (light)	Day 82	
9	Transplant 5 transgenic plants per event to soil (small pots)	Day 89	4 regenerated transgenic events 
10	Transplant 4 transgenic plants per event to soil (large pots)	Day 110	
11	Pollinate transgenic ear	Day 154	3 transgenic fertile events
12	Harvest seed	Day 194	

Fig. 1. Time line and efficiency cascade for production of fertile transgenic plants from Hi II immature embryos transformed with *A. tumefaciens*. After cocultivation, all immature embryo explants are subcultured through resting and selection (Steps 2–5). Picking of putative transgenic events begins at about Day 52 (Step 6) but can continue for as long as 10 wk after infection. We recover an average of five putative, independent transgenic events (bialaphos-resistant calli) from 100 *Agrobacterium*-infected immature embryos. Ninety percent of these Hi II putative transgenic callus events are successfully regenerated to plants and grown to maturity in the greenhouse. We typically cross four transgenic plants per event to produce more than 50 seeds from three of every four transgenic events taken to seed.

2.3. Stock Solutions

2.3.1. Vitamins and Phytohormones

1. N6 vitamin stock (1000X): 1.0 g/L thiamine HCl, 0.5 g/L pyridoxine HCl, 0.5 g/L nicotinic acid, 2.0 g/L glycine. Store in 50-mL aliquots in Falcon tubes at -20°C . Thaw one tube at a time and store at 4°C .
2. MS vitamin stock (1000X): 0.5 g/L thiamine HCl, 0.5 g/L pyridoxine HCl, 0.05 g/L nicotinic acid, 2.0 g/L glycine. Store 50-mL aliquots in Falcon tubes at -20°C . Thaw one tube at a time and store at 4°C .
3. 2,4-Dichlorophenoxy acetic acid (2,4-D): weigh 0.25 g 2,4-D in a fume hood, and dissolve in 1 N KOH (10 mL) on low heat. When dissolved, bring up to 250 mL final volume with ddH₂O water. Store at 4°C in Duran bottle.

2.3.2. Antibiotics and Selective Agents

1. Chloramphenicol (Sigma): 25 mg/mL stock in ddH₂O. Store filter-sterilized 0.05-mL aliquots in Eppendorf tubes at -20°C .
2. Kanamycin sulfate (Sigma): 10 mg/mL stock in ddH₂O. Filter-sterilize through a 0.2- μm membrane (Fisher Scientific). Store 0.25-mL aliquots in Eppendorf tubes at -20°C .
4. Spectinomycin sulfate (Sigma): 100 mg/mL stock in ddH₂O. Filter-sterilize and store 0.05-mL aliquots in Eppendorf tubes at -20°C .
5. Bialaphos: dissolve 100 mg of bialaphos (Duchefa) in 100 mL ddH₂O. Filter-sterilized stock solution (1 mg/mL) is stored at 4°C for up to 4 mo in 50-mL Falcon tubes (see **Note 3**).
6. Glufosinate: dissolve 100 mg of glufosinate ammonia (Sigma) in 100 mL of ddH₂O. Stock solution (1 mg/mL) is filter-sterilized and stored in 50-mL Falcon tubes at 4°C .
7. Cefotaxime: dissolve 1.0 g of cefotaxime (Phytotechnology Laboratories) in 5 mL ddH₂O. Store filter-sterilized stock solution (200 mg/mL) in 0.25-mL aliquots and store at -20°C .
8. Vancomycin: dissolve 1.0 g of vancomycin (Phytotechnology Laboratories) in 5 mL ddH₂O. Store filter-sterilized stock solution (200 mg/mL) in 0.25-mL aliquots at -20°C (see **Note 4**).

2.3.3. Other

1. Acetosyringone (AS): stock solution (100 mM) is prepared by dissolving 0.392 g of AS (Sigma) in 10 mL of dimethyl sulfoxide (DMSO) and then diluting this 200 mM solution 1:1 with ddH₂O before filter-sterilizing. Store 0.5-mL aliquots in Eppendorf tubes at -20°C .
2. L-Cysteine: dissolve 100 mg/mL L-cysteine (Sigma) in ddH₂O and filter-sterilize. Use the same day (see **Note 5**).
3. Silver nitrate: dissolve 0.85 g silver nitrate (Fisher) in 100 mL of ddH₂O. Filter-sterilize the stock solution (50 mM) and store at 4°C for up to 1 yr in a foil-wrapped container to avoid exposure to the light.

2.4. Culture Media

2.4.1. For *Agrobacterium*

1. YEP medium (**20**): 5 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl₂ (Fisher), pH 6.8, with NaOH. Add Bacto-agar (15 g/L) to prepare solid YEP medium. For growing pTF101.1 in EHA101, the final antibiotic concentrations in YEP are: 50 mg/L kanamycin (disarmed Ti plasmid pEHA101), 100 mg/L spectinomycin (binary vector plasmid pTF101.1), and 25 mg/L chloramphenicol (*Agrobacterium* chromosome). Pour into 100 × 15-mL Petri plates.

2.4.2. For Maize (see Note 6)

1. Infection (liquid) medium: 4 g/L N6 salts (Sigma; **21**), 1 mL/L N6 vitamin stock, 1.5 mg/L 2,4-D, 0.7 g/L L-proline, 68.4 g/L sucrose, and 36 g/L glucose; adjust pH to 5.2 using 1 N KOH. Filter-sterilize and store at 4°C. Acetosyringone (AS) is added immediately prior to use for a final concentration of 100 μM.
2. Cocultivation medium: 4 g/L N6 salts, 1.5 mg/L 2,4-D, 0.7 g/L L-proline, 30 g/L sucrose, and 3 g/L Gelrite; adjust pH to 5.2 using 1 N KOH. Then 1 mL/L filter-sterilized N6 vitamin stock, 5 μM silver nitrate, 100 μM AS, and 300 mg/L L-cysteine are added after autoclaving when medium has cooled. This medium should be made fresh and used within 4 d.
3. Resting medium: 4 g/L N6 salts, 1.5 mg/L 2,4-D, 0.7 g/L L-proline, 30 g/L sucrose, 0.5 g/L 2-(4-morpholino)-ethane sulfonic acid (MES), and 8 g/L purified agar; adjust pH to 5.2 using 10 N KOH. Then 1 mL/L filter-sterilized N6 vitamins, 100 mg/L cefotaxime, 100 mg/L vancomycin, and 5 μM silver nitrate are added after autoclaving when medium is cooled.
4. Selection medium I: 4 g/L N6 salts, 1.5 mg/L 2,4-D, 0.7 g/L L-proline, 30 g/L sucrose, 0.5 g/L MES, and 8 g/L purified agar; adjust pH to 5.8 using 10 N KOH. Then 1 mL/L filter-sterilized N6 vitamins, 100 mg/L cefotaxime, 100 mg/L vancomycin, 5 μM silver nitrate, and 1.5 mg/L bialaphos are added after autoclaving when medium is cooled.
5. Selection medium II: the same as selection medium I except that bialaphos concentration is increased to 3 mg/L.
6. Regeneration medium I: 4.3 g/L MS salts (Sigma; **22**), 1 mL/L MS vitamin stock, 100 mg/L myoinositol, 60 g/L sucrose, 3 g/L Gelrite; adjust pH to 5.8 using 1 N KOH. Filter-sterilized 6 mg/L glufosinate ammonia and 100 mg/L cefotaxime are added after autoclaving when medium is cooled.
7. Regeneration II: 4.3 g/L MS salts, 1 mL/L MS vitamin stock, 100 mg/L myoinositol, 30 g/L sucrose, 3 g/L Gelrite; adjust pH to 5.8 using 1 N KOH.

2.5. Other Supplies and Reagents

1. Sterilizing solution: 50% commercial bleach (5.25% hypochlorite), 1 drop of surfactant Tween-20 per liter.
2. Redi-Earth: cat. no. 10-2030-1, Hummert (Earth City, MO).
3. Sunshine Universal Mix SB300: Fosters (Waterloo, IA).

4. Greenhouse flat with drainage holes (holds 32 small pots): Hummert, cat. no. 11-3000-1.
5. Small pot (6.4 cm² each in four-packs): Hummert, cat. no. 11-0300-1.
6. Humi-dome (plastic, transparent): Hummert, cat. no. 14-3850-1.
7. Large pot for Hi II (2-gallon nursery pot with four drainage holes): Hummert, cat. no. 14-9606-1.
8. Large pot for B73 pollen donor plants (3-gallon nursery pot with four drainage holes). Hummert, cat. no. 14-9612-1.
9. Osmocote Plus 16-8-12 (controlled release fertilizer tablets with trace elements): Hummert, cat. no. 07-6450-1.
10. Calcium/magnesium solution (Dr. C. Block, USDA-ARS, North Central Regional Plant Introduction Station, Ames, IA): first make two separate stock solutions.
 - a. Stock #1: 720 g/L of Ca(NO₃)₂·4H₂O.
 - b. Stock #2: 370 g/L of MgSO₄·7H₂O (Epsom salts).
 - c. To prepare working solution, add 5 mL each of stock #1 and stock #2 into 1 gallon (~4 L) of H₂O (see **Note 7**).
11. Miracle Gro Excel 15-5-15 (water-soluble fertilizer supplemented with calcium and magnesium, 1.5 g per 10 L water): Hummert, cat. no. 07-5660-1.
13. Marathon™ (restricted-use pesticide for aphid control): Hummert, cat. no. 01-1118-1.
14. Whitemire yellow monitoring cards (for fungus gnat control): Hummert, cat. no. 01-2700-1.
15. Shoot bags: Lawson, cat. no. 217, Lawson Bags (Northfield, IL).
16. Striped (red or green) tassel bags: Lawson, cat. no. 404.
17. Plain (brown) tassel bags: Lawson, cat. no. 404.
18. Vent tape (1 inch): Fisher, cat. no. 19-027-761

3. Methods

3.1. Growing Donor Plants for Immature Embryo Production

1. Seed germination is conducted in the greenhouse (see **Notes 8** and **9**).
2. Fill a plastic four-pack with Sunshine Universal Mix SB300. Water until the soil is completely wet.
3. Bury one corn seed 2.5 cm deep in the middle of each pot. Place the four-packs in a greenhouse flat with drainage holes under a plastic Humi-dome for 1 wk to avoid excessive evaporation of moisture from the soil.
4. Seeds should germinate in approximately 4 d. Check moisture daily; water the plants only when the soil is dry.
5. After 9 d, or just before the plants reach the top of the dome, remove the plastic dome and continue to water as needed.
6. After approximately 2 wk, transplant each seedling (approx 4–5-leaf stage) into a large pot.
7. To begin, half fill a 2-gallon (7.6-L) pot with Sunshine Universal Mix, and add 1 tablet (15 g) of Osmocote Plus. Add Universal Mix to 80% of the pot (~5 cm from top edge of pot; see **Notes 10** and **11**).

8. To transplant, move corn plantlet with soil adhering to the root ball from the small pot and lay it on the unwetted soil surface in the middle of the big pot. Press the roots into the soil and bury them to a depth that keeps the plant from falling over as it grows (see **Note 12**).
9. Drench the soil by filling until the water level reaches to the top edge of the pot. Let drain completely, and drench again (see **Notes 13** and **14**).
10. Prior to internode elongation, place one more tablet of Osmocote Plus fertilizer onto the soil surface of each pot. For B73 plants, a third controlled release fertilizer application may be needed (see **Notes 15** and **16**).
11. Fifty-five to 60 d after germination, embryo donor plants are sib-pollinated for producing embryo donor ears for transformation.
12. Cover any emerging ears with shoot bags to prevent contamination of the silks before controlled pollination (see **Note 17**).
13. After the silks have been visible (under the shoot bag) for 1 to 2 days, cut them and the top inch of the cob off to prepare a uniform surface of silks for pollination the next day (see **Notes 18** and **19**). Use white or light yellow pollen from sibling Hi II plants for pollination (see **Note 20**).
14. Cover the pollinated silks with a plain pollination bag and label with the plant ID and cross date. Ears can be harvested 9 (summer) to 13 (winter) d after pollination. The ideal size of immature embryos for transformation should be between 1.5 and 2 mm (see **Note 21**).

3.2. Preparation of *Agrobacterium* Culture for Infection

1. The vector, pTF101.1, in strain EHA101 is maintained on solid YEP with antibiotics at 4°C for 1 mo (mother plate) before it is refreshed from long-term, -80°C glycerol stocks.
2. To initiate a maize transformation experiment, streak *A. tumefaciens* from a 4°C mother plate to solid YEP with antibiotics and grow for 3 d at 19°C (see **Note 22**).
3. On the day of infection, inoculate one loop full (3 mm) of this bacteria culture in 5 mL infection medium supplemented with 100 μ M AS in a 50-mL Falcon tube.
4. Shake the culture gently for 2 to 5 h by taping it horizontally to a Vortex Genie (Fisher) platform set on low speed (approx 75 rpm)

3.3. Ear Sterilization and Embryo Dissection

1. While the *A. tumefaciens* is being precultured, surface-sterilize the maize ears. To begin, cut off and discard the top 1 cm of a dehusked maize cob (silk end). Insert the tip of a straight-nosed forceps into this end to secure the cob for aseptic manipulation during embryo dissection. In a laminar flow bench, place up to four ears in a sterile Mason jar, with forceps handles protruding.
2. Add approx 700 mL (enough to cover ears) of sterilizing solution. During the 15- to 20-min disinfection, occasionally tap the Mason jar on the bench surface to dislodge air bubbles. Pour off bleach solution and rinse the ears three times in generous amounts of sterilized water (see **Notes 23** and **24**).

3. In a large (150 × 15-mm) sterile Petri plate, cut off the top 1 to 2 mm of kernel crowns using a new (sharp) scalpel blade (see **Note 25**).
4. Insert the end of a sharpened spatula straight down (do not insert at an angle or you may split the embryo in half) between the endosperm and pericarp at the basipetal side of the kernel (toward the bottom of the cob).
5. Pop the endosperm out of the seed coat by gently wiggling the spatula. (If you dislodge the kernel from the cob instead, you have inserted the spatula too deeply.) This exposes the untouched embryo, which is then gently coaxed onto the spatula tip and transferred directly to liquid infection medium.

3.4. Infection, Cocultivation, and Resting

1. Dissect up to 100 immature zygotic embryos directly into a 2-mL Eppendorf tube filled with bacteria-free infection medium (with 100 μ M AS).
2. Remove this first wash using a 1-mL pipetman, and wash embryos a second time with the same medium (1 mL).
3. After removing the final wash, add 1 mL of precultured *A. tumefaciens* suspension (adjusted to OD₅₅₀ = 0.30 to 0.40 using a spectrophotometer) to the embryos. The tube is gently inverted (not vortexed) 20 times before resting it upright for 5 min on the bench with embryos submerged.
4. After infection, transfer embryos, along with the *A. tumefaciens* suspension to the surface of the cocultivation medium (300 mg/L L-cysteine) using a 1-mL pipet tip. (Cut off to enlarge the bore size.) Carefully pipet off any excess *A. tumefaciens* suspension surrounding the embryos using an uncut tip.
5. Orient infected embryos scutellum side up with the aid of a dissecting scope. Wrap plates with vent tape and incubate at 20°C (dark) for 3 d.
6. Transfer all embryos from the cocultivation medium to resting medium (35 embryos per plate). Wrap plates with vent tape and incubate at 28°C (dark) for 7 d (see **Note 26**).

3.5. Selection for Putative Transgenic Callus Events

1. After the 1-wk resting period, transfer all embryos to selection I medium (1.5 mg/L bialaphos) to begin selection. Plates are wrapped with parafilm and incubated at 28°C (dark).
2. Two weeks later, selection pressure is enhanced by transferring embryos to selection II medium (3 mg/L bialaphos) for two subcultures of 2 wk each.
3. About 6 wk after infection, a rapidly growing, embryogenic sector of callus emerges on a subset of infected embryos while no further callus proliferation, and in some cases browning, occurs on the majority of the other embryos. Each of these proliferating calli is considered an independent putative transgenic event (see **Note 27**).
4. Subculture each putative event to its own plate of selection medium II for maintenance and naming.
5. Molecular biological analyses such as Southern or Northern blot hybridization or histochemical β -glucuronidase (GUS) assays can be performed at this stage.

3.6. Regeneration of Transgenic Plants

1. With the aid of a 40X dissecting microscope, up to 15 pieces (4 mm) of bialaphos-resistant, embryogenic callus (stocked embryos suspended in a friable callus matrix) from one independent transformation event are transferred to a single plate of regeneration medium I (see **Note 28**). Plates are wrapped with vent tape and incubated at 25°C (dark) for 2 to 3 wk.
2. After this maturation period, the majority of somatic embryos are swollen, opaque, and white, and, from some pieces, the coleoptile is already emerging. Again using a dissecting microscope, transfer these mature somatic embryos (approx 12 pieces) to the surface of regeneration medium II for germination in the light (25°C, 80 to 100 $\mu\text{E}/\text{m}^2/\text{s}$ light intensity, 16:8-h photoperiod) and wrap the plates with vent tape.
3. Each somatic embryo sprouts leaves and roots on regeneration medium II within 1 wk, and plantlets are ready for transfer to soil within 10 d.

3.7. Transplanting and Acclimation

1. In a laminar flow hood, use sterile forceps to transfer plantlets (a good-sized plantlet measures about 5 cm) from the Petri plate to the soil surface of a small pot filled with Redi-Earth (see **Note 29**).
2. Any medium still clinging to the roots is removed, and plants are handled with extreme care to avoid breaking off the leaf. Premade tags identifying the source of each plantlet are inserted into each small pot at this time and accompany the plant through to maturity in the greenhouse.
3. Plantlet roots are gently pressed into the soil and covered. Place small pots into greenhouse flat with drainage holes. Thoroughly soak the flat with a gentle stream of water so as not to dislodge the transplants.
4. Place the flat in the growth chamber and cover it with a Humi-dome in which one ventilation hole has been cut (see **Note 30**).
5. Flats should not need water for 48 h if thorough soaking was done at transplant. After that, water individual plants only as needed.
6. Remove Humi-dome when plants are tall enough to touch it, and 1 wk later move the flat from the growth chamber to the greenhouse (see **Note 31**).

3.8. Greenhouse Care of Transgenic Plants

1. Once transgenic plants have been moved to the greenhouse, continue to monitor soil moisture on a per plant basis. Water only if dry, using a watering can with a well-defined spout.
2. Transgenic plantlets are fertilized once using liquid Miracle Gro Excel 15-5-15 water-soluble fertilizer before transplanting to big pots.
3. A plantlet is ready to be transplanted to a big pot if soil adheres to its root ball when it is lifted out of the small pot. Plantlets are generally over 16 cm tall at this stage.
4. Follow **Steps 7 to 9** in **Subheading 3.1.** for transplanting transgenic plants from small pots to big pots.

5. After they are transplant to big pots, plants are watered the first five times with 50 ppm Miracle Gro Excel 15-5-15.
6. Follow **Steps 7 and 10** in **Subheading 3.1.** for fertilization instructions.
7. Molecular analysis can be performed at this stage.

3.9. Transgenic Seed Production

1. We cross all our R_0 female transgenic plants by pollinating them with non-transgenic donor pollen.
2. To provide nontransgenic donor pollen to pollinate transgenic ears, begin by planting two donor seeds twice per week (four per week) as soon as the first transgenic material is transferred to the light on regeneration I medium. Planting rate is doubled as the bulk of events for a project begin to go to the light (**Step 2** of **Subheading 3.6.**; see **Note 32**).
3. Follow **Steps 12 to 14** in **Subheading 3.1.** for controlled pollination.
4. Striped pollination bags are used for differentiating transgenic and nontransgenic crosses in the greenhouse, and all bags are labeled with the cross ID (female plant ID $\times$ male plant ID) and date using a thick, black, permanent marker.
5. Tassels of all R_0 transgenic plants are bagged as soon as tassels emerge to minimize transgenic pollen flow in the greenhouse. In addition, transgenic plants are grown in a separate room from the nontransgenic, pollen donor plants.
6. After pollination, watering is continued as needed until 21 to 25 d later, at which time watering is stopped altogether, and plants are moved to a dry-down area.
7. To further aid in cob dry-down, lift the pollination bag off the ear 15 d post pollination. Ten to 15 d later, pull down the husks to facilitate further drying of the kernels (see **Notes 33** and **34**).
8. Forty to 45 d after pollination, harvest the seed. Seed is inventoried and securely stored in the cold (0–3°C, 60% relative humidity).

4. Notes

1. We have also recovered transgenic events using construct pTF101.1 and constructs derived from the pTF101.1 vector (i.e., pTF102; **6**) in strains LBA4404 (**23**) and GV3101::pMP90 (**24,25**) and in strains AGL-0 and AGL-1 (**26**) and C58 Z707 (**27**).
2. F_1 seeds planted year round in the greenhouse are produced in the field by pollinating Hi II parent A ears with Hi II parent B pollen. These parent lines can be obtained from the Maize Genetics Cooperation Stock Center (<http://w3.aces.uiuc.edu/maize-coop/>) and are increased and maintained in our field by sib-pollination when feasible. Hi II plants generally take 60 d (depending on the season) to flower in the greenhouse.
3. To date we have used bialaphos from Shingyo Sangyo (Tokyo, Japan), but it is no longer available from this source. In the future we will be ordering from Gold Bio Technology (Duchefa Plant Biotechnology Products) at <http://www.goldbio.com>.
4. If these two formulations of cefotaxime and vancomycin are mixed together before you add them to the medium, they will form a precipitate, so add separately to the cooled medium.

5. The L-cysteine stock appears to come out of solution if left overnight at 4°C. Thus we make this stock fresh each time we make cocultivation medium.
6. All media described in **Subheading 2.4.2.** (except liquid infection medium) use 100 × 25-mm vented lid Petri plates (Fisher) and are poured to a volume of 32 plates/L. Media 1 to 5 are after Zhao et al. (19) with the addition of cysteine (300 mg/L) to cocultivation medium and the use of cefotaxime and vancomycin instead of carbenicillin for counterselection of *A. tumefaciens* after cocultivation. Regeneration medium I is after Armstrong and Green (28) and McCain et al. (29). All media are dried thoroughly before storage at room temperature in the dark.
7. The stock solutions should be made separately rather than adding both salts to one bottle of water. If they are not made separately, gypsum will immediately be formed.
8. Corn plants in our greenhouse are placed in pots on the ground beginning 2 wk after transplant to large pots. Our greenhouse operates on a 16:8-h photoperiod. The average temperature is 28°C (day) and 21°C (night). The light intensity (230 $\mu\text{E}/\text{m}^2/\text{s}$ at 3.5 ft above ground) was measured in February on a slightly overcast day and therefore does not factor in any additional sunlight.
9. To provide a steady flow of immature embryos for *Agrobacterium* transformation experiments, 10 Hi II F₁ seeds are planted twice per week to guarantee 15 ears per week to the lab.
10. If pots are filled with too little soil, they will dry out too quickly between waterings. Conversely, if they are filled too full, thorough drenching is prevented at watering.
11. Marathon, for aphid control, can be added at this stage as part of the transplant step. If white flies or fungus gnats hover over pots, yellow monitoring sheets can be used to reduce or eliminate the insects.
12. The soil should cover all the roots. Be sure to plant the young plants deep enough or they will tip over when they grow taller and before their brace roots have formed.
13. Normally, no watering is needed for 1 wk if plants are drenched thoroughly at transplant (7–14 d later they are watered as needed). Check the soil moisture daily by lifting the pot and feeling its weight. Water when the pot can be lifted easily with one hand. Watering may not be needed if only the surface layer of soil is dry. If the water pressure from the hose sends soil flying out of the pot, turn it down to avoid leaving excess dirt on the greenhouse floor after each watering.
14. Pay special attention to small plants. Young plants will begin to develop a strong root system if not overwatered.
15. Plants will need more water as they mature. During the summer, fully grown plants may need to be watered twice a day.
16. Calcium deficiency may occur at this stage (or earlier or later). Plants develop rippled edges and unpigmented patches or lesions on the leaves and may also be severely stunted. In the worst cases, the leaves in the whorl wrap tightly around each other and eventually rot. To treat plants for calcium deficiency, water them

once every 2 wk with calcium/magnesium solution. As a precautionary measure, continue treating plants for several weeks after they have stopped showing symptoms.

17. Be sure shoot bags are firmly pulled over ear shoots; otherwise they will fall off, and your emerging silks will be “contaminated” with unwanted pollen.
18. If spent silks on embryo donor ears become moldy, clean scissors with ethanol before cutting back ears as a preventative measure.
19. If silks have emerged for as long as a week, they can still be cut back and recovered with the shoot bag; the fresh “stubs” can be pollinated the next day.
20. Do not use old and crystallized yellow pollen because it is not viable, although pollen color will vary depending on genotype. Be careful with B73 tassels; they are easy to break off accidentally, and maneuvering the tall plant to obtain pollen can be difficult.
21. Ears harvested from the greenhouse (or field) are stored in their husks and pollination bags in a larger plastic bag at 4°C. If there are not enough freshly harvested ears for an experiment, ears stored from Friday through Sunday, or Tuesday through Thursday, are used for experiments on Monday and Friday, respectively. We have not experimented with ears stored for longer than 5 d.
22. We occasionally grow *Agrobacterium* for 2 d at 28°C in preparation for an experiment. In initial side-by-side comparisons, the 19°C/3 d combination resulted in higher but not significantly different transformation efficiencies than 28°C/2 d, so we have continued to use the former (B. Frame, unpublished data). T-DNA transfer machinery was reported to function optimally at 19°C compared with 28°C (30).
23. If you are surface-sterilizing a large number of ears, save time and resources by using a preautoclaved 4-L beaker, which will hold up to 20 ears at a time. We often do this when processing up to 27 field ears a day during August.
24. Greenhouse ears can be surface-sterilized for as little as 15 min, but we always use the full 20 min when sterilizing field ears, from which we routinely encounter more problems with fungal or bacterial contamination in experiments.
25. Intermittent resterilization of all utensils used for dissection is accomplished using a Steriguard 350 bead sterilizer (Inotech Biosystems, Rockville, MD).
26. After 3 d on freshly prepared cocultivation medium containing 300 mg/L cysteine, embryos smaller than 1.5 mm are often flaccid and slow to produce callus. Nevertheless, transfer all embryos from cocultivation medium to resting and from resting to selection media, regardless of their appearance.
27. Using the protocol described, at an average efficiency of 5%, we expect to recover five independent, bialaphos-resistant calli per 100 infected embryos. When using Hi II germplasm in which we recover few to no escapes (events that do not carry the *bar* selectable marker gene), we calculate transformation efficiency as: (number of bialaphos-resistant calli recovered/total number of embryos infected) $\times$ 100.
28. Glufosinate ammonium contains the same active ingredient (phosphinothrycin) as bialaphos. We use it instead of bialaphos during regeneration because it is less

expensive and more readily available, although in our experience, glufosinate must be used at twice the concentration of bialaphos to achieve the same effect. Imposing continued selection pressure during this first regeneration step (whether glufosinate or bialaphos) is effective because nontransgenic callus does not form mature somatic embryos on this medium. Thus only callus containing the *bar* gene is advanced to the light after this final in vitro selection step.

29. Typically, plantlets on each plate germinate at different rates. After transplanting the large plantlets, smaller plantlets in the same Petri plate are returned to the light until they too are large enough for transplant to soil.
30. We use a Conviron (Controlled Environments Limited, 590 Berry St. Winnipeg, Manitoba, Canada) growth chamber for this stage. The conditions are: 16:8-h photoperiod; 350 $\mu\text{E}/\text{m}^2/\text{s}$ light intensity (plant height of 30 cm) with a combination of fluorescent and incandescent bulbs; 26°C (day) and 22°C (night)].
31. If the greenhouse is in a different building than the laboratory and the outdoor temperature is below freezing or very cold, special care is needed during this step. Cover the flat with a Humi-dome, wrap it in a plastic garbage bag, and transport it to the greenhouse in a preheated vehicle.
32. To prevent peak load problems, not all transgenic events from one DNA construct are taken to the greenhouse at once.
33. If seeds are contaminated by fungus, use 70% ethanol to clean the surface before storage.
34. Common pests found in our greenhouse are aphids (on the tassels or on the underside of leaves), spider mites (on the leaf back), and thrips (on the leaf whorl). We spray for mites or thrips once a month, or as needed, using Floramite™, Pylon™, Akari™, Avid™, or Samite™ for spider mite control and Conserve™ for thrip control (all from Hummert). Fungus gnats are another common pest and often become a problem when plants are overwatered. Place yellow monitoring cards (to which the air-borne gnats stick) around the greenhouse to control them. Another disease that we see although infrequently is smut. Smutted plants are immediately discarded. To limit disease onset, the greenhouse must be kept clean. The floor should be frequently swept or sprayed clean, the drain hole always left unclogged, and fallen and dead leaves still clinging to the plants should be removed. Garbage should be emptied once a week. Mice moving in from the field in the fall will feed on maize seed (transgenic or otherwise). To solve this problem, we set out mice traps baited with peanut butter each autumn.

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Indica Rice (*Oryza sativa*, BR29 and IR64)

Karabi Datta and Swapan Kumar Datta

Summary

Rice is the world's most important food crop. Indica-type rice provides the staple food for more than half of the world population. To satisfy the growing demand of the ever-increasing population, more sustained production of indica-type rice is needed. In addition, because of the high per capita consumption of indica rice, improvement of any traits including its nutritive value may have a significant positive health outcome for the rice-consuming population. Rice yield productivity is greatly affected by different biotic stresses, like diseases and insect pests, and abiotic stresses like drought, cold, and salinity. Attempts to improve resistance in rice to these stresses by conventional breeding through introgression of traits have limited success owing to a lack of resistance germplasm in the wild relatives. Gene transfer technology with genes from other sources can be used to make rice plants resistant or tolerant to insect pests, diseases, and different environmental stresses. For improving the nutritional value of the edible endosperm part of the rice, genes for increasing iron, β -carotene, or better quality protein can be introduced in rice plants by genetic engineering. Different crops have been transformed using various gene transfer methods, such as protoplast transformation, biolistic, and *Agrobacterium*-mediated transformation. This chapter describes the *Agrobacterium*-mediated transformation protocol for indica-type rice. The selectable marker genes used are hygromycin phosphotransferase (*hpt*), neomycin phosphotransferase (*nptII*), or phosphomannose isomerase (*pmi*), and, accordingly, the selection agents are hygromycin, kanamycin (G418), or mannose, respectively.

Key Words: Indica-type rice; *Agrobacterium*-mediated transformation; *Agrobacterium tumefaciens*; genetic engineering; marker gene.

1. Introduction

Rice (*Oryza sativa* L.), especially indica-type rice, is the most important staple food crop for tropical and to some extent subtropical Asia. The world population is expanding, and the availability of land for cultivation is declining because of urbanization. As a result, the rate of population growth is exceeding

the rate of food grain production. To feed the world population with nutritious food, the staple and sustained production of high-quality food is needed. Improving rice cultivars through genetic engineering (transformation) by introducing and expressing a specific gene(s) that is not available in that cultivar could be an important strategy for overcoming food shortages.

The transgenic method may benefit farmers and consumers in different ways. It offers a powerful tool to improve and protect productivity by protecting plants from biotic and abiotic stresses. Pesticide or herbicide application may be reduced by making plants with built-in protection by genetic engineering, such as Bt-containing or herbicide-resistant crops. Reducing pesticide use can also have a positive impact on the environment and the farmers' health. Transgenic methods may be used to improve the micronutrient content of the edible part of the food crop.

Different systems have been developed for the generation of transgenic plants. Popularly used transformation methods are based on biological vectors (*Agrobacterium*-mediated), physical techniques (particle bombardment), and chemically mediated techniques (PEG/electroporation-mediated protoplast transformation). The first genetically engineered fertile indica type rice was reported in 1990; only the selectable marker gene had been inserted (1). Many agronomically important genes have now been transferred into indica-type rice for plant protection from biotic stresses (2,3), for protection from abiotic environmental stresses (4–6), and for improvement of the nutritional quality of the rice endosperm (7–10). Some of the transgenic indica-type rice has been field evaluated and found to have an excellent performance.

The basic requirement for production of transgenic plants are target tissues competent for plant regeneration and a suitable selection system for selecting only the transformed cells, leading to subsequent regeneration of transgenic plants.

Several selectable marker genes like *nptII*, *hpt*, and *bar* (*ppt*) have been used in cereal transformation (1,11–14). For indica-rice transformation, using *hpt* as the marker gene works well, although there is public concern about the use of antibiotic resistance genes in crop transformation. Alternatively, a marker-free transgenic event or a new POSITECH™ selection strategy that involves mannose (carbohydrate) as the selecting agent can be used (8,13). This selection system may lead to better public acceptability of transgenic rice.

Agrobacterium-mediated transformation has been extensively used in plant systems, particularly rice (13–17). In this chapter a detailed protocol for the *Agrobacterium*-mediated gene transfer method for indica rice transformation is described. Scutellum-derived embryogenic calli have been used as initial explants for transformation. The efficiency of indica rice transformation varies from genotype to genotype; in general, japonica rice is at least 10 times more responsive than indica rice. Traditional indica rice is often found to be more tissue culture responsive than the improved IRRI variety (e.g., IR64; Table 1).

Table 1
Development of Indica Rice Cultivars with Agronomic Traits
Using *Agrobacterium*-Mediated Transformation at the International Rice Research Institute (IRRI)

Transformed variety	Agro strain used	Gene(s) used	Selectable marker gene	Trait	No. of independent lines	References
Basmati 122	LBA4404	<i>Chi11</i>	<i>hpt</i>	ShB tolerance	15	16
Tulsi	LBA4404	<i>Chi11</i>	<i>hpt</i>	ShB tolerance	45	16
Vaidehi	LBA4404	<i>Chi11</i>	<i>hpt</i>	ShB tolerance	27	16
Nang Huong	LBA4404	<i>CryIAb/CryIB</i>	<i>ppt</i>	Insect resistance	12	21
Cho Dao						
Mot Bui	LBA4404	<i>CryIAb/CryIB</i>	<i>ppt</i>	Insect resistance	5	21
BR29	LBA4404	<i>OsDREB1A</i>	<i>hpt</i>	Abiotic stress	26	Datta et al. (in preparation)
	LBA4404	<i>OsDREB1B</i>	<i>hpt</i>	Abiotic stress	14	Datta et al. (in preparation)
	LBA4404	<i>AtDREB1A</i>	<i>hpt</i>	Abiotic stress	10	Datta et al. (in preparation)
IR64	LBA4404	<i>psy + crtI</i>	<i>pmi</i>	Carotenoid biosynthesis	10	Datta et al. (in preparation)
BR29	EHA101	<i>psy + crtI</i>	<i>pmi</i>	Carotenoid biosynthesis	221	Datta et al. (in preparation)
	LBA4404	<i>psy + crtI</i>	<i>pmi</i>	Carotenoid biosynthesis	157	Datta et al. (in preparation)
	LBA4404	<i>psy + crtI</i>	<i>hpt</i>	Carotenoid	34	13
BR-28	LBA4404	<i>psy + crtI</i>	<i>hpt</i>	Carotenoid	6	13
IR68144	LBA4404	<i>psy + crtI</i>	<i>hpt</i>	Carotenoid	25	13

ShB, sheath blight; *hpt*, hygromycin phosphotransferase; *ppt*, phosphinotrycin acetyltransferase; *pmi*, phosphomannose isomerase.

2. Materials

2.1. *Agrobacterium tumefaciens* Strains and Selectable Markers

1. *A. tumefaciens* strain LBA4404 or EHA101. The gene of interest is introduced into the binary vector using standard molecular biological techniques.
2. The binary vector (pCacar, pCAMBIA) containing the *nptII* gene as a selectable marker driven by the CaMV35S promoter (13).

2.2. Culture Media for *Agrobacterium tumefaciens*

1. AB medium (14): 3 g/L K_2HPO_4 , 1 g/L $NaH_2PO_4 \cdot H_2O$, 1 g/L NH_4Cl , 300 mg/L $MgSO_4 \cdot 7H_2O$, 150 mg/L KCl, 10 mg/L $CaCl_2 \cdot 2H_2O$, 2.5 mg/L $FeSO_4 \cdot 7H_2O$, 10 g/L glucose, 30 g/L agar. Autoclave, and then cool to 55°C before adding 50 mg/L kanamycin, or the selective agent depending on the vector used.
2. AAM medium (14; see Table 2).
3. Luria-Bertani (LB) medium: 10 g/L Bacto-tryptone (Difco), 5 g/L Bacto yeast extract (Difco), 10 g/L NaCl, and 12 g/L bacto agar (for solid medium). Autoclave, and then cool to 55°C before adding 20 mg/L rifampicin and 50 mg/L kanamycin (or appropriate selective agent depending on the vector).

2.3. Plant Material

Oryza sativa: indica-type rice cultivars, BR29 (popular rice of Bangladesh), IR64 (IRRI elite variety); immature embryos (panicles collected 7–10 d after anthesis) or healthy mature seeds from the mentioned cultivars.

2.4. Sterilizing Solution

1. 70% Ethanol.
2. 50% Chlorox with 1–2 drops of Tween-20.
3. Sterile Distilled Water.

2.5. Stock Solution

1. 2, 4-Dichlorophenoxy acetic acid (2,4-D; Sigma): 20 mg/100 mL. Dissolve the powder in a few drops of 0.1 N KOH, make the final volume with ddH₂O, and store at 4°C.
2. Cefotaxime (Invitrogen): 100 mg/mL. Filter-sterilize. Store at –20°C.
3. 10 mM $MgSO_4$ solution: in water, sterilize by autoclaving. Store at 4°C.
4. Acetosyringone (3', 5', dimethoxy-4'-hydroxy-acetophenone, Sigma-Aldrich): 40 mg/mL (200 mM). Dissolve the powder in a few drops of dimethyl sulfoxide (DMSO; Sigma), and make the required volume with ddH₂O. Filter-sterilize. Store at 4°C.
5. Kinetin (Sigma): 20 mg/100 mL. Dissolve the powder in a few drops of 1 N HCl, and then add ddH₂O to make volume. Store at 4°C.
6. NAA (Sigma): 20 mg/100 mL. Dissolve the powder in a few drops of 0.1 N NaOH, make the final volume with ddH₂O, and store at 4°C.
7. Hygromycin: (Calbiochem): 50 mg/mL in water. Filter-sterilize. Store at –20°C.

Table 2
AAM Medium^a

Components	Amount (mg/L)
Macronutrients	
CaCl ₂ ·2H ₂ O	150.0
MgSO ₄ ·7H ₂ O	250.0
NaH ₂ PO ₄ ·H ₂ O	150.0
KCl	2950.0
Micronutrients	
KI	0.75
H ₃ BO ₃	3.0
MnSO ₄ ·H ₂ O	10.0
ZnSO ₄ ·7H ₂ O	2.0
Na ₂ MoO ₄ ·2H ₂ O	0.25
CuSO ₄ ·5H ₂ O	0.025
CoCL ₂ ·6H ₂ O	0.025
Iron composition	
Na ₂ EDTA	37.3
FeSO ₄ ·7 H ₂ O	27.8
Vitamins	
Nicotinic acid	0.5
Pyridoxine HCl	0.5
Thiamine HCl	1.0
Glycine	2.0
Inositol	100.0
Others	
L-glutamine	876.0
Aspartic acid	266.0
Arginine	174.0
Casamino acid	500.0
Sucrose	68.5 g/L
Glucose	36 g/L
Acetosyringone	200 μM
pH-5.2, Filter sterilization	

^aMix components before adjusting pH. Filter-sterilize.
See **ref. 14**.

Table 3
MS Medium (Modified)^a

Name of stock solution	Component	Quantity (g/L)	Stock vol for 1 L medium (mL)	Final concentration (mg/L)
MS1	NH ₄ NO ₃	82.5	20	1650.0
	KNO ₃	95.0		1900.0
MS2	MgSO ₄ ·7H ₂ O	37.0	10	370.0
	MnSO ₄ ·4H ₂ O	2.23		22.3
	ZnSO ₄	1.058		10.6
	CuSO ₄ ·5H ₂ O	0.0025		0.025
MS3	CaCl ₂ ·H ₂ O	44.0	10	440.0
	KI	0.083		0.83
	CoCl ₂ ·6H ₂ O	0.0025		0.025
MS4	KH ₂ PO ₄	17.0	10	170.0
	H ₃ BO ₃	0.62		6.2
	Na ₂ MoO ₄ ·2H ₂ O	0.025		0.25
MS5	FeSO ₄ ·7H ₂ O	2.785	10	27.85
	Na ₂ EDTA·2H ₂ O	3.725		37.25
Vitamins	Nicotinic acid	10.0 mg/100 mL	5	0.5
	Pyridoxine HCl	10.0 mg/100 mL		0.5
	Thiamine HCl	20.0 mg/100 mL		1.0
	Glycine	40.0 mg/100 mL		2.0
Hormone	2,4-D	10 mg/100 mL	20	10.0
Myoinositol				100.0
Sucrose/maltose				30,000.0
Agar				8000.0
pH	5.6–5.8			

^aAll the inorganic salts are from Merck and the rest are from Sigma.

2.6. Media for Plant Tissue Culture

1. MS-2-4-D medium: MS salts and vitamins (see [Table 3; 18](#)), 300 mg/L casamino acid, 2 mg/L 2,4-D, and 8 g/L agar, pH 5.8. Autoclave.
2. N6-AS medium for infiltration (liquid) and cocultivation (solid): N6 salts (see [Table 4; 19](#)), MS vitamins (see [Table 3](#)), 300 mg/L casamino acid, 2 mg/L 2,4-D, 30 g/L sucrose, 10 g/L glucose, 9 g/L agar (for cocultivation medium), pH 5.2. Autoclave, and cool to 55°C before adding acetosyringone (200 μM).
3. Selection media: MS salts, MS vitamins, 300 mg/L casamino acid, 2 mg/L 2,4-D, 500 mg/L cefotaxime (add after autoclaving), 8 g/L agar, pH 5.8.

Table 4
N6 Medium (Modified)^a

Stock solution	Component	Quantity (g/L)	Stock vol for 1 L medium (mL)	Final concentration (mg/L)
N61	KNO ₃	141.50	20	2830.0
N62	MgSO ₄ ·7H ₂ O	18.5	10	185.0
	MnSO ₄ ·4H ₂ O	0.44		4.4
	ZnSO ₄	0.15		1.5
	(NH ₄) ₂ SO ₄	46.3		463.0
N63	CaCl ₂ ·2H ₂ O	16.6	10	166.0
N64	KI	0.08	10	0.8
	KH ₂ PO ₄	40		400
	H ₃ BO ₃	0.16		1.6
N65	FeSO ₄ ·7H ₂ O	2.785	10	27.85
	Na ₂ EDTA·2H ₂ O	3.725		37.25
Vitamins	Nicotinic acid	10.0 mg/100 mL	5	0.5
	Pyridoxine HCl	10.0 mg/100 mL		0.5
	Thiamine HCl	20.0 mg/100 mL		1.0
	Glycine	40.0 mg/100 mL		2.0
Hormone	2,4-D	10 mg/100 mL	20	10.0
Myoinositol				100.0
Sucrose/maltose				30,000.0
Agar				8000.0
pH	5.6–5.8			

^aAll the inorganic salts are from Merck and the rest are from Sigma.

- For selection with the *hpt* gene, add 30 g/L sucrose, 50 to 100 mg/L hygromycin (depending on cultivar used, add after autoclaving)
 - For selection with the *pmi* gene, add 20 g/L sucrose and 15 g/L mannose for the first round of selection; add 10 g/L sucrose and 20 g/L of mannose for the second round of selection, and add 5 g/L sucrose and 25 g/L mannose for the third round of selection.
- Regeneration medium (MSKN): MS salts, MS vitamins, 2 mg/L kinetin, 1 mg/L NAA, 300 mg/L casamino acid, 50 mg/L cefotaxime, 30 g/L sucrose, 10 g/L sorbitol, 2.5 g/L gelrite. pH 5.8. Autoclave.
 - Rooting medium (MSO): MS salts, MS vitamins, 30 g/L sucrose, 2.5 g/L Gelrite, pH 5.8. Autoclave.
 - Yoshida's culture solution (see [Table 5; 20](#)), pH 5.0, freshly prepared.
 - Sandy-loam paddy soil.
 - A mixture of fertilizer [2.5 g of (NH₄) SO₂, 1.25 g of P₂O₅, 0.75 g of K₂O per pot (15 × 20 cm)].

Table 5
Yoshida Culture Solution^a

Component	Stock concentration (g/10 L)
MgSO ₄ ·7H ₂ O	3240.0
NH ₄ NO ₃	914.0
CaCl ₂ ·2H ₂ O	886.0
K ₂ SO ₄	714.0
NaH ₂ PO ₄ ·2H ₂ O	403.0
H ₃ BO ₃	300.0
FeCl ₃ ·6H ₂ O	77.0
MnCl ₂ ·4H ₂ O	15.0
ZnSO ₄ ·7H ₂ O	0.35
CuSO ₄ ·5H ₂ O	0.31
(NH ₄) ₆ Mo ₇ O ₂₄ ·4H ₂ O	0.74

^aTake 10 mL from stock of each component for every 8 L final volume of culture solution.

3. Methods

3.1. Establishment of Embryogenic Calli from Immature or Mature Embryos

1. Remove the hull from immature or mature seeds (see **Note 1**).
2. Surface sterilize the dehulled seeds (mature or immature) with 70% alcohol for 1 min and then chlorox solution by shaking at 60 rpm for 30 min. Wash the seeds three times with sterile deionized water.
3. Isolate the embryos from the sterile seeds and place individually in a Petri dish (100 × 15 mm) containing 20 mL MS-2,4-D medium (25 embryos/dish). The scutellar tissue side should be upward, since embryogenic calli growth occurs with this orientation. Incubate the embryos in the dark at 25°C.
4. After 3 to 4 d, cut off the emerging shoots and roots, and subculture the remaining scutellar tissues onto fresh medium with same orientation. Incubate the tissues in the dark at 25°C (see **Note 2**).
5. After 2 to 3 wk, yellowish white soft embryogenic calli develop on the surface of scutella.

3.2. Preculture Agrobacterium Strain

1. Day 1: Take a loop full of culture from a glycerol stock culture of *Agrobacterium* strain EHA101 or LBA4404 harboring a binary vector and streak on AB medium (or LB solid medium) containing 20 mg/L rifampicin and 50 mg/L kanamycin (or antibiotic appropriate for vector used).
2. Incubate the plates in the dark at 28°C for 2 d (see **Note 3**).

3.3. Preculture of Calli (3 Days prior to Transformation)

1. Day 1: select embryogenic calli (size approx 1.3–3 mm diam.) from 4- to 5-wk old culture, and transfer to fresh MS-2,4-D medium (approx 100 calli/dish).
2. Incubate at 28°C in the dark for 3 d.

3.4. Culture of *Agrobacterium* Strain

1. Day 3: transfer three to four well-grown *Agrobacterium* colonies to 50 mL of AAM (or LB liquid) medium containing appropriate selective antibiotic (20 mg/L rifampicin and 50 mg/L kanamycin) in a 250-mL conical flask (see **Note 4**).
2. Let the *Agrobacterium*-culture grow in liquid, shaking at 250 rpm for 20 to 30 h (depending on growth of the *Agrobacterium*) at 28°C.

3.5. Transformation

1. Day 4: add acetosyringone (200 μ M) in *Agrobacterium* suspension and keep shaking at 250 rpm for 2 h at 28°C.
2. Centrifuge the bacterial culture in liquid medium at 3500g for 30 min at 10°C.
3. Harvest the bacteria in 20 mL MgSO₄ (10 mM) in a 50-mL centrifuge tube (see **Note 5**).
4. Take the OD measurement of the *Agrobacterium* suspension (600 nm).
5. Centrifuge the harvested bacteria at 3500g for 30 min at 10°C. Discard the supernatant.
6. Resuspend the bacterial pellet in small volume of liquid N6-AS infiltration medium by sucking up and down with a pipet.
7. Make the final volume to adjust the OD of *Agrobacterium* suspension to 1 with N6-As medium ([19,21](#)).
8. Put the embryogenic calli in the bacterial suspension in a Petri dish (50 × 18 mm).
9. For vacuum infiltration, place the Petri dish open in a vacuum desiccator for 10 min.
10. Let stand for another 20 min, and then pipet out the bacterial suspension from the embryogenic calli.
11. Absorb the excess *Agrobacterium*-infection medium from the calli using sterile paper towels or filter paper.
12. Transfer calli to N6-AS solid cocultivation medium and incubate the plates for 3 d at 28°C under dark condition.

3.6. Proliferation of Calli

1. Transfer the calli on MS-2,4-D medium with 250 mg/L cefotaxime for proliferation of the calli (see **Note 6**).
2. Keep the calli in the dark at 25°C for 10 d.

3.7. Selection

1. Place the calli in the first selection medium, containing 250 mg/L cefotaxime and selecting agent depending on the selectable marker gene for transformation and keep in the dark for 2 wk at 25°C (see **Note 7**).

2. Transfer the surviving healthy embryogenic calli from the first selection to the second selection medium containing 250 mg/L cefotaxime and the selecting agent. Keep in the dark for another 2 wk.
3. Subculture the surviving embryogenic calli for the next round of selection. Keep in dark for 2 wk at 25°C.

3.8. Regeneration

1. Transfer the healthy surviving embryogenic calli to MSKN regeneration medium. Keep the culture in the dark for 20 d (see **Note 8**).
2. Harvest the emerging shoots and transfer again to fresh regeneration medium (MSKN) in a 25-mL conical flask. Place in the light at 27°C with a 16-h photoperiod (110 $\mu\text{mol}/\text{m}^2/\text{s}$). Keep for 10 to 20 d (see **Note 9**).
3. Transfer the healthy shoots to the rooting medium (MSO).

3.9. Transfer to Soil

1. After 10 to 14 d, when a well-developed root system is observed, gently remove the plantlets from the culture medium and wash out the medium from the roots using water (see **Note 10**).
2. Transfer the plantlets individually into styrofoam boards with holes, and place over a plastic tray filled with Yoshida culture solution (20).
3. Place the tray in a phytotron or greenhouse (containment facility), 14-h photoperiod (160 $\mu\text{mol}/\text{m}^2/\text{s}$), 70 to 95% relative humidity, and 29°C/21°C day/light temperature condition. Keep for 21 d.
4. Transfer the plants with vigorous root systems into pots containing soil, water (1 L per pot and 500 mL each day until maturity), and sufficient fertilizer after initial fertilizer (see **Subheading 2.5.8.**). Add 2.5 g $(\text{NH}_4)_2\text{SO}_4$ at the initial stage of flowering.
5. Harvest panicles when 85% of grains have already turned straw gold in color. It requires around 110 to 120 d for seed harvesting. In general, we obtain 600 to 1000 seeds per plant in BR29, whereas IR64 produces around 400 to 600 seeds (see **Note 11**).

4. Notes

1. As the immature or mature seeds are used from greenhouse grown plants, dehulling is necessary to avoid the chance of contamination.
2. Removal of shoots and roots allows better embryogenic calli development.
3. Always keep Petri dishes in upside down condition, as proper growth of bacteria takes place in hanging position.
4. Culture of *Agrobacterium* can also be done by streaking on solid AB medium, but the growth of *Agrobacterium* is better and quicker in liquid medium.
5. In case of *Agrobacterium* grown in solid medium, harvest directly from the solid medium to MgSO_4 solution.
6. After transformation there is no need to grow *Agrobacterium* together with rice calli; it inhibits the growth of calli. Cefotaxime is used to kill the bacteria.

7. For hygromycin phosphotransferase as the selectable marker, use hygromycin as the selective agent; for phosphomannose isomerase as the selectable marker, a combination of sucrose and mannose can be used.
8. Take care of those embryogenic calli developing faster and appearing healthier than the rest. Transfer them earlier for regeneration. Faster developing calli produce healthy plants.
9. To regenerate the transformed line of independent event, harvest only one shoot/callus. Discard the callus once the shoot is harvested from it.
10. Washing the medium from the roots reduces the chances of contamination with bacteria and fungi, which have adverse effects on plant growth.
11. Sometimes the first-generation seed setting is low owing to tissue culture effect.

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Japonica Rice Varieties (*Oryza sativa*, Nipponbare, and Others)

Philippe Hervé and Toshiaki Kayano

Summary

Agrobacterium-mediated transformation of rice is now used in many laboratories worldwide. Several protocols have been developed and fine-tuned for particular genotypes, including commercial genotypes, making use of either mature seeds or immature embryos as target tissue for *Agrobacterium* infection. In this chapter, we describe a rapid and user-friendly protocol based on mature seeds that can deliver transgenic rice plantlets within 2 mo. The protocol described is based on the use of the *nptII* selectable marker gene. The tissue culture steps rely on *Agrobacterium* infection of whole young seedlings and on the induction and proliferation of highly embryogenic tissue. Importantly, we have validated the robustness and reliability of this protocol at a high-throughput scale for several japonica genotypes. We also provide some key features that can be further explored for the fine-tuning of this transformation protocol for any other genotype with a particular emphasis on the importance of tissue handling and subculture sequence.

Key Words: Rice; *Oryza sativa*; *Agrobacterium*; genetic engineering; transformation; tissue culture; growth regulators; transgenic.

1. Introduction

Among cereals, rice has become a model plant in functional genomics thanks mainly to its small genome and the availability of efficient gene transfer methods. Rice and maize have been the first Monocot species to be successfully transformed with *Agrobacterium* (1,2). Whereas maize transformation still faces several technical bottlenecks and genotype range limitation, protocols for rice genetic engineering developed over the last decade are reliable, efficient, and relatively easy to implement. The japonica rice cultivar Nipponbare, whose genome has been sequenced and which is most commonly used in functional genomics, is among the genotypes that are most amenable to

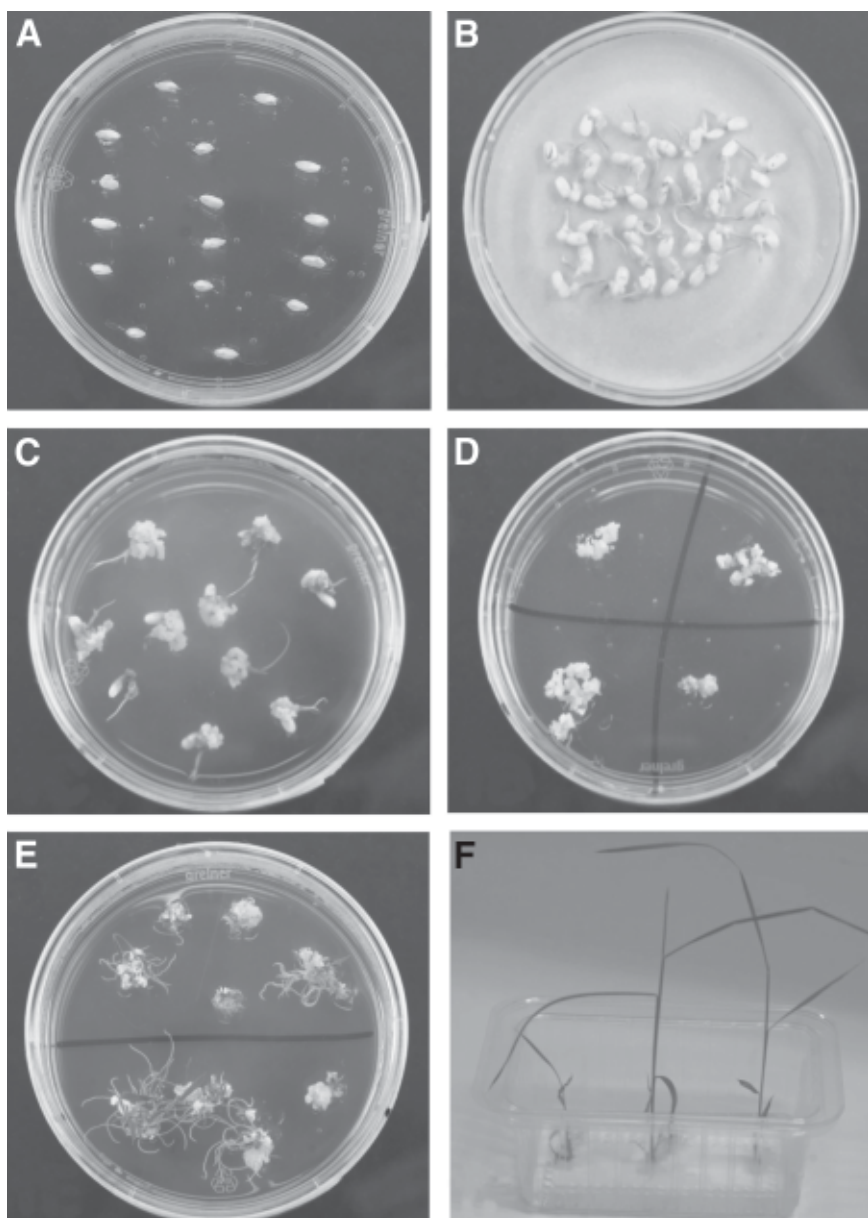


Fig. 1. Steps of the transformation protocol. **(A)** Induction. Seeds are placed into the medium, embryo-side upward. **(B)** Cocultivation. **(C)** Selection. Resistant lines with proliferative embryogenic calli. **(D)** Regeneration after 2 wk. **(E)** Regeneration after 3 wk. **(F)** Plantlet development.

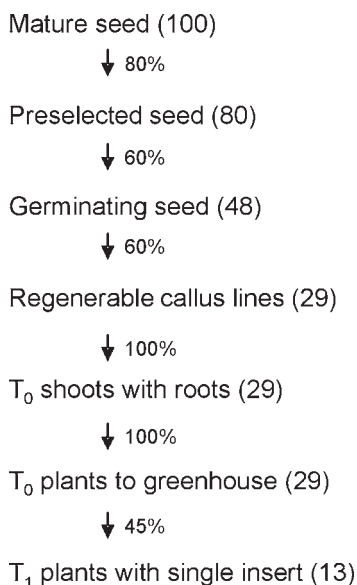


Fig. 2. Efficiency flow of the protocol with Nipponbare. For each individual step, the success rate is shown. From 100 mature seeds, 13 independent single-insert T_0 events can be obtained within 2 mo.

transformation (3,4). However, several research groups have successfully established reproducible protocols for a broad range of japonica and indica genotypes including commercial cultivars (5–7). Common features of these protocols identify key parameters of the tissue culture steps and provide the basis for implementing any new protocol for other genotypes. It is also well known that both mature seeds and immature embryos of rice can be used as targets for *Agrobacterium* infection and that the scutellum or scutellum-derived tissues are responsive for *Agrobacterium* cocultivation. For genotypes that are highly responsive to tissue culture, mature embryos are generally preferred for practical reasons such as growth room space limitation. More recalcitrant genotypes may, however, require immature embryos for efficient transformation.

In this chapter, we describe the steps for a reproducible *Agrobacterium*-mediated transformation protocol for mature seeds that has been successfully used with a number of japonica genotypes (Fig. 1). In addition, we have been successful with at least two different selectable marker genes and we provide a protocol based on the public domain selectable marker gene neomycin phosphotransferase II (*nptII*) originating from the *E. coli* transposon Tn5. The major advantage of this user-friendly protocol is the short timeframe of the tissue culture steps on growth regulator-containing media, which reduces

both sterility of the T_0 plants and unwanted tissue culture-induced somaclonal variation. We have established a standard procedure with the cultivar Nipponbare that results in about 60% of infected seedlings yielding independent polymerase chain reaction (PCR)-positive events (Fig. 2). This protocol has proved to be very robust and in a high-throughput system generates over 10,000 independent transgenic events per person per year. We provide some key features that can be further explored for improving the application of available protocols to recalcitrant genotypes. We also discuss the importance of factors such as high-quality seeds and reliable growth chamber conditions for this protocol. In our respective laboratories, we have shown that a higher temperature (up to 32°C) and continuous light significantly speed up the procedure (8). The protocol described has been successfully applied to a number of japonica rice varieties, including Nipponbare, Nakdong, Suweon330, Anjungbyeol, Taipei309, Cheniere, and Katy. The same protocol has also been applied to indica rice Basmati 370 (9) with hygromycin selection and carbenicillin (500 mg/L).

2. Materials

2.1. Plant Material and Growth in a Greenhouse

1. Mature seeds of rice from field-grown or greenhouse-grown plants are stored according to standard seed storage procedures (15% relative humidity and 15°C). Importantly, high-quality seeds (without visible fungus contamination or damage) are required to avoid or reduce any microbial contamination during the first step of tissue culture.
2. Greenhouse conditions for growing T_0 plants are 25 to 28°C (day) and 18 to 24°C (night) with a 12-h/12-h light/dark regime (200–250 $\mu\text{E}/\text{m}^2/\text{s}$) and 70% relative humidity.
3. The plants are grown in clay-rich (15%) soil mixture (ASEF® pond ground) and watered/fertilized by an automated sub-irrigation system (two cycles of 2 h).
4. Mix 1 fertilizer (Peters Professional® 10+52+10 + LibFer® Fe-EDDHA 1.625 g/L + BMS MicroNutrients NV Chelal® Mg 1.25% v/v) is used for 2 wk after transplanting.
5. Mix 2 fertilizer (Peters Professional 15+10+30 + LibFer Fe-EDDHA 1.625 g/L + Chelal Mg 1.25% v/v) is used from the third week and applied until early physiological seed maturity, after which plants are only watered with water until seed harvest.

2.2. Agrobacterium Strains and Plasmids

Agrobacterium tumefaciens strain LBA4404 containing a standard binary vector is used (10). The selectable marker functional cassette contains the coding sequence of the neomycin phosphotransferase II (*nptII*) gene (originating from the *E. coli* transposon Tn5), flanked by a promoter from the rice ubiquitin gene and a terminator sequence of the *Agrobacterium* octopine synthase gene (*ocs*). *Agrobacterium* cells are stored in a glycerol stock (30%) at –80°C.

2.3. Media Preparation

2.3.1. Media

1. The composition of the different plant media is described in [Table 1](#). Tissue culture salts, plant growth regulators, vitamins, sugars, acetosyringone, gelling agents, and antibiotics are purchased from Sigma Aldrich. BACTO™ casamino acids is purchased from Difco. Media are autoclaved for 15 min at 121°C. All heat-sensitive chemicals (plant growth regulators and antibiotics) are filter-sterilized and added after autoclaving as described in [Table 1](#). Media are stored at 8°C for a maximum of 2 wk. All agar-based media are poured into Petri dishes (100 × 20 mm) except for R07, which is poured into translucent plastic boxes. Petri dishes are sealed with Micropore™ (3M) tape.
2. AB medium ([11](#)): 1 g/L NH₄Cl, 0.3 g/L MgSO₄·7 H₂O, 0.15 g/L KCl, 0.01 g/L CaCl₂, 2.5 mg/L FeSO₄·7H₂O, 3 g/L KH₂PO₄, 1 g/L NaH₂PO₄, 5 g/L D-glucose monohydrate solidified with 15 g/L Difco Bacto Agar.

2.3.2. Stock Solutions

1. 2,4-Dichlorophenoxyacetic acid (2,4-D): 1 mg/mL stock solution. Prepare by dissolving in 1 M KOH (4% v/v), dilute with deionized water, and filter-sterilize (0.22-μm pore size). Store at 4°C for 1–2 mo.
2. Naphtalene acetic acid (NAA): 1 mg/mL stock solution. Prepare by dissolving in 1 M NaOH and dilute with deionized water; filter-sterilize (0.22-μm pore size). Store frozen in aliquots at –20°C.
3. Kinetin: 1 mg/mL stock solution. Prepare by dissolving in 1 M NaOH, dilute with deionized water, and filter-sterilize (0.22-μm pore size). Store frozen in aliquots at –20°C.
4. Abscissic acid (ABA): 5 mg/mL stock solution. Prepare by dissolving in dimethyl sulfoxide (DMSO). Store in aliquots at –20°C.
5. 200 mg/mL Cefotaxime; 100 mg/mL Vancomycin; and 100 mg/mL G418 stock solutions. Prepare by dissolving in deionized water and sterilize by filtration (0.22-μm pore size). Store frozen in aliquots at –20°C.
6. Acetosyringone: 2 M stock solution. Freshly prepare by dissolving in DMSO.

2.4. Tissue Culture Conditions

All tissue cultures except cocultivation are incubated under continuous light (50 μE/m²/s, using Philips TLD 58W33 fluorescent tubes) at 32°C. Cocultivation is performed at 25°C (dark).

3. Methods

3.1. Mature Seed Sterilization (see [Note 1](#))

1. Select healthy seeds (without visual symptoms of fungus contamination or damage).
2. Dehusk seeds using a rice seed husker (Kett, model TR120) and select seeds with intact embryos.

Table 1
Composition of the Media

Component (final concentration)	R00	R01	R02	R03	R04	R05	R06	R07
N6 salts/vitamins	1X	1X	1X	1X	1X			
MS salts/vitamins						1X	1X	1X
L-proline (mg/L)	2878			2878	500			
Casamino acids (mg/L)	300	300	300	300	300	2000	2000	
Sucrose (g/L)	30	68.5	30	30	30	30	30	30
Sorbitol (g/L)						30	30	
D-glucose monohydrate (g/L)		36	10					
2,4-D (mg/L) ^a	2		2	2				
NAA (mg/L) ^a					1	0.02	0.02	
ABA (mg/L) ^a					5			
Kinetin (mg/L) ^a					2	2	2	
Gelrite (g/L)	4		4					4
Agarose type I (g/L)				7	7	10	7	
Cefotaxime (mg/L) ^a				100	100	100	100	
Vancomycin (mg/L) ^a				100	100	100	100	
G418 disulfate (mg/L) ^a				35	20	20	20	
Acetosyringone (μM) ^a		100	100					
pH	5.8	5.2	5.2	5.8	5.8	5.8	5.8	5.8
Sterilization	A	F	A	A	A	A	A	A

^aFilter-sterilized chemical added after autoclaving. A, autoclaved; F, filter-sterilized.

3. Put about 200 dehusked seeds into a sterile 50-mL tube.
4. Wash the seeds with 70% ethanol for 1 min.
5. Remove the ethanol and wash the seeds once with sterile deionized water.
6. Add a solution of 3% sodium hypochlorite (containing 1 drop of Tween-20 per 50 mL), and shake the tubes gently for 30 min using a Rotator drive system or equivalent.
7. Remove the sodium hypochlorite solution and wash at least six times with sterile deionized water.

3.2. Explant Preparation

1. Place 12 to 16 seeds onto induction medium R00. Importantly, the seeds are buried into the medium with the embryo side upward. The embryo is located at the surface of the medium in order to maximize the contact surface between the scutellum tissue and the medium (Fig. 1A).
2. Petri dishes are incubated under continuous light at 32°C for 5 d (see Note 2).

3.3. *Agrobacterium* Infection (see Note 3)

1. *Agrobacterium* strains are grown from a glycerol stock on AB medium with appropriate antibiotics for 3 d at 28°C.
2. One loop of *Agrobacterium* culture is scraped from the 3-d-old plates and resuspended in 40 mL infection medium R01 in a sterile 50-mL tube. The *Agrobacterium* suspension is used without a preinduction step.
3. Adjust the *Agrobacterium* suspension to an OD₆₀₀ from about 0.05 to about 0.1 (see Note 4).
4. Select 5-d-old germinated seedlings showing clear swelling of the scutellum.
5. Transfer whole seedlings into the *Agrobacterium* suspension and shake by gently inverting the tube for about 2 min.
6. Remove the *Agrobacterium* suspension and quickly blot the seedlings onto sterilized filter paper in order to remove excess *Agrobacterium* suspension.
7. Transfer 50 seedlings onto a sterilized filter paper previously moistened with 0.5 mL of liquid infection medium placed on solid cocultivation medium R02 (Fig. 1B). Petri dishes are incubated at 25°C in the dark for 3 d.

3.4. Selection of Transformed Embryogenic Tissues

1. After 3 d of cocultivation, gently wash the whole seedlings four to six times with sterile deionized water, the last wash containing 250 mg/L cefotaxime (see Note 5).
2. Quickly blot the seedlings onto sterilized filter paper and transfer about 12 to 16 whole seedlings onto selection medium R03. Petri dishes are incubated under continuous light at 32°C for 2 to 3 wk (Fig. 1C; see Notes 2, 6, and 7).

3.5. Regeneration of Transformed Plantlets (see Note 8)

1. For each original infected seedling, transfer resistant embryogenic calli (white/yellow proliferative calli arising from the scutellum) onto regeneration medium R04. Petri dishes are incubated under continuous light for 1 wk. At least four

independent transgenic events (embryogenic calli arising from four different seedlings) are cultivated per Petri dish.

2. Surviving calli are transferred onto regeneration medium R05 for 1 wk. At least four independent transgenic events (embryogenic calli) are cultivated per Petri dish (**Fig. 1D**).
3. Surviving calli are transferred onto regeneration medium R06 for another 2 wk. Two independent transgenic events are cultivated per Petri dish (**Fig. 1E**).
4. Plantlets arising from the calli are isolated and transferred onto germination medium R07 and incubated under a continuous light regime for 1 or 2 wk in order to promote shoot and root development (**Fig. 1F**).

3.6. Transplanting and T_1 Seed Production

1. Carefully wash the roots of well-developed 8- to 10-cm plantlets.
2. Transplant each plantlet by placing it in a 4- to 5-cm deep hole in prewatered soil mixture in 12-cm-diameter pots.
3. Place the pots under a plastic Humi-dome for 1 wk (see **Note 9**).
4. Remove the pots from the Humi-dome and grow plants in the greenhouse for about 10 to 16 wk until harvesting of T_1 seeds (see **Note 9**). On average, more than 50% of the T_0 plants produce at least 50 T_1 seeds.

4. Notes

1. We recommend testing several seed batches because the efficiency of the protocol depends on the choice of high-quality seeds. For transformation experiments, mature seeds from field-grown plants are highly recommended. Other sterilization methods can be applied; however, we recommend increasing either the concentration of sodium hypochlorite (e.g., up to 6%) or the time of sterilization (e.g., up to 60 min) rather than using a mercury chloride treatment, which may decrease seed responsiveness.
2. Increased temperature (32°C instead of 28°C) and continuous light conditions significantly increase the responsiveness of seedlings and the proliferation rate of embryogenic calli arising from the scutellum. A weekly subculture is also recommended for recalcitrant genotypes. These conditions are also very important for the first induction step but are not necessarily a prerequisite for success.
3. Transformation efficiency is higher using strains EHA101 or EHA105 than strain LBA4404 (data not shown). However, strains EHA101 and EHA105 give rise to more multicopy insertions, and the number of single-insert plants per 100 infected seedlings is actually higher using the *Agrobacterium* strain LBA4404 than using either strains EHA101 or EHA105. With strain LBA4404, the percentage of single-copy inserts among the primary transgenic plants is typically in the range of 30 to 45% (data not shown). In addition, we have tested several constitutive promoters (data not shown) and demonstrated that the low concentration (35 mg/L) of the selective agent (G418) as described in this protocol is efficient with the different promoters tested.

4. We have shown that the *Agrobacterium* suspension density can be very low using either EHA or LBA4404 strains. We advise an OD₆₀₀ range from 0.05 to 0.08.
5. The washing step of infected seedlings after cocultivation may be omitted if there is no visible *Agrobacterium* growth after 3-d cocultivation. In addition, the remaining part of the seed may be removed from the swollen scutellum before washing in order to reduce the risk of *Agrobacterium* overgrowth. The last wash may also be done with R03 medium without gelling agent containing 250 mg/L cefotaxime.
6. Gelling agents other than agarose can be used in combination with other selectable markers. For example, Gelrite (4 g/L) can be used with hygromycin (50 mg/L) when using an *hpt*-based selectable marker. With an *nptII*-based selectable marker, we have chosen agarose (type I) as a gelling agent because it also significantly increases the responsiveness of the tissue, which is particularly relevant with genotypes more recalcitrant than Nipponbare. Importantly, G418 cannot be used with Gelrite.
7. The addition of coconut water (10% v/v) to selection medium can be particularly important during the selection step in order to increase the overall efficiency of more recalcitrant genotypes, but it is not a prerequisite for success.
8. For several genotypes, R04, R05, and R06 regeneration media can advantageously be replaced by a single growth regulator-free medium containing high sucrose concentration (60 g/L). In such a case, cultures are incubated for 2 wk on that medium and then transferred directly onto the R07 medium for further plantlet development.
9. The use of a Humi-dome may be omitted if the relative humidity of greenhouse or growth chamber is well-controlled. Importantly, the root system of the in vitro T₀ plantlets should be well-developed for successful transplanting. The timing for heading and harvesting of T₁ seeds can vary significantly (10–16 wk) because of seasonal and transgene effects. Weekly visual inspection of the T₀ plants is required.

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Rye (*Secale cereale* L.)

Fredy Altpeter

Summary

Rye (*Secale cereale* L.) is one of the most recalcitrant plant species for tissue culture and genetic transformation. Embryogenic rye callus loses its ability to regenerate plants quickly in response to high density of *Agrobacterium* and other stressors. The cocultivation of *Agrobacterium* and rye immature embryos in liquid medium facilitated washing of the cultures to avoid *Agrobacterium* overgrowth and allowed a high throughput. More than 40 independent transgenic plants were regenerated with one to four Southern-positive, independent events from 100 inoculated immature embryos. *Agrobacterium* strain AGL0 supported stable integration of a constitutive *nptII* selectable marker expression cassette into the genome of rye inbred line L22, as indicated by regeneration of plantlets on paromomycin-containing culture medium, Southern blot, Western blot, and the analysis of T-DNA::plant DNA boundary sequences. Transgenic plants were phenotypically normal and fully fertile, which might be a consequence of the short time in tissue culture.

Key Words: Rye; *Secale cereale* L.; cereal transformation; liquid cocultivation medium; *Agrobacterium*-mediated gene transfer; *npt II*; cereal transformation; inbred lines; AGL0.

1. Introduction

Genetic transformation is a powerful tool for crop improvement and requires the development of reproducible and efficient protocols supporting gene transfer into plant cells and the regeneration of normal and fertile plants from transgenic *in vitro* cultures. Rye is known as one of the most recalcitrant species for regeneration from tissue cultures and genetic transformation (1). Injection of plasmid DNA directly into floral tillers describes an early attempt to transform rye genetically (2). Convincing molecular evidence for the generation of a few transgenic rye plants was presented for the first time in 1994, following biolistic gene transfer into embryogenic callus (1). Rye is the only

cross-pollinated species among the small grain cereals, resulting in a significant genotypic variability within a cultivar. This variability also reduces the reproducibility of tissue culture response from rye cultivars (3). Selfing is naturally prevented in rye by an effective gametophytic self-incompatibility mechanism (4). Self-fertile forms have been found in several rye populations and are routinely used for developing inbred lines in rye-hybrid breeding programs (5).

In the early transformation experiments, cross-pollinating rye populations were used (2). The identification of inbred lines displaying a good regeneration response from tissue cultures (3) and the optimization of gene transfer, selection, and tissue culture parameters allowed the development of a reproducible and efficient biolistic transformation protocol for rye (6). Biolistic gene transfer is a highly effective method for molecular improvement of cereals, including rye (7). Compared with *Agrobacterium*, biolistic gene transfer is usually successful in a wider range of genotypes, and cotransfer of multiple genes is facilitated (8). *Agrobacterium*-mediated gene transfer also offers potential advantages over biolistic gene transfer. These include preferential integration of T-DNA's into transcriptional active regions (9,10), and elimination of selectable marker genes is facilitated by frequent integration of cotransformed T-DNA's into separate chromosomes (11,12). Cereals 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 (13), wheat (14), barley (15), and rye (16). The transfer of T-DNA and its integration into the plant genome is influenced by the vector plasmid (15), the bacteria strain (11,17), the addition of *vir* gene-inducing synthetic phenolic compounds (18), culture media composition, culture conditions (19) and osmotic stress treatments (20) during and before *Agrobacterium* infection, the plant genotype, the explant, and the tissue culture protocol, as well as the suppression and elimination of *Agrobacterium* after cocultivation (21).

This chapter describes a detailed protocol for stable *Agrobacterium*-mediated genetic transformation of rye. This genetic transformation protocol has supported the generation of transgenic rye plants with single and defined T-DNA inserts (16) with one to four Southern-positive, independent events from 100 inoculated immature embryos.

2. Materials

2.1. *Agrobacterium* Strain and Plasmid

Electroporation (22) was used to introduce the binary vector pJFnptII (23) into *Agrobacterium tumefaciens* strain AGL0 (24). pJFnptII encodes the selectable marker gene *nptII* under control of the constitutive corn ubiquitin promoter with the first intron (25) and the 35-S terminator in the pPZP111 vector backbone (26).

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- μ m 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 monosulfate, 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 monosulfate.

2.3. Donor Plant Production to Obtain Tissue Culture Explants

1. Plants of rye (*Secale cereale* L.) spring inbred line L22 (Lochow-Petkus, Bergen, Germany) are grown in the greenhouse at 12°C to 16°C during the night and 16°C to 20°C during the day with a 12/12-h light/dark cycle until the end of tillering. Then a 16/8-h light/dark cycle is used after tillering to produce immature caryopses (see **Note 1**).
2. Topsoil, peat, and sand were mixed in a 3:1:1 ratio for plant growth, and plants were fertilized biweekly with Peters fertilizer (20:20:20 with micronutrients; St. Louis, MO), following the manufacturer's recommendations. Light intensity of at least $360\ \mu\text{E}/\text{m}^2/\text{s}$ at plant height was maintained with sodium vapor lights. When immature embryos were approximately 2 mm in length, corresponding to developmental stage 3 (27) (see **Note 2**), immature caryopses were harvested and sterilized for obtaining immature embryo explants.

2.4. Rye Tissue Culture Media

1. 70% (v/v) Ethanol.
2. Sterilizing solution: sodium hypochlorite solution (2.4% active chlorine) supplemented with a surfactant (0.1% [w/v] of Tween-20).
3. Vitamin stock: Murashige and Skoog vitamin mixture 1000X (PhytoTechnology Laboratories, Shawnee Mission, KS). Dissolve 10.31 g of the premixed vitamins in 100 mL water. Filter-sterilize (0.2 μ m) and store 1-mL aliquots at -20°C .
4. 2,4-Dichlorophenoxyacetic acid (2,4-D): 2 mg/mL stock solution. Prepare by dissolving in a minimum amount of warm (60°C) 1 M KOH and make to volume with water. Store 1-mL aliquots at 4°C .
5. Acetosyringone 100 mM stock solution: dissolve 196.2 mg of acetosyringone in 10 mL DMSO.
6. Paromomycin sulfate (PhytoTechnology Laboratories) 50 mg/mL stock solution: dissolve in water, filter-sterilize (0.2 μ m), and store 1-mL aliquots at -20°C .
7. Timentin (PhytoTechnology Laboratories) 150 mg/mL stock solution: dissolve in water filter sterilize (0.2 μ m) and use immediately.
8. Basic medium (BM): 4.3 g basal Murashige and Skoog salts (GIBCO BRL, cat. no. 11117-874; 28), 1 mL/L Murashige and Skoog 1000X vitamin stock, 100 mg/L casein hydrolysate, 500 mg/L glutamine.

9. Callus induction medium (CIM): BM plus 30 g/L sucrose, 1.25 mL/L 2,4-D stock solution, pH 5.8, 3.0 g/L Phytagel.
10. Osmotic treatment medium (OTM): BM plus 30 g/L sucrose, 3.0 mL/L 2,4-D stock solution, 72.9 g/L mannitol, pH 5.8.
11. Cocultivation medium liquid (CCML): BM plus 15 g/L sucrose, 15 g/L glucose, 3.0 mL/L 2,4-D stock solution, pH 5.2, 2.0 mL/L acetosyringone stock solution (see **Notes 3** and **4**).
12. Cocultivation medium solid (CCMS): CCML solidified with 3.0 g/L Phytagel.
13. Subculture medium (SCM): CIM plus 1 mL/L timentin stock solution.
14. Shoot regeneration medium I (SRM I): BM plus 30 g/L sucrose, pH 5.8, 5.0 g/L agarose (type I), 1.0 mL/L timentin stock solution, 0.6 mL/L paromomycin sulfate stock solution (see **Note 5**).
15. Shoot regeneration medium II (SRM II): BM plus 30 g/L sucrose, pH 5.8, 5.0 g/L agarose (type I), 1 mL/L timentin stock solution, 1.0 mL/L paromomycin sulfate stock solution (see **Note 5**).

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 after autoclaving (at a medium temperature of less than 50°C) as concentrated, filter-sterilized solutions. Acetosyringone is added after autoclaving (at a medium temperature of less than 50°C). 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 immature caryopses for 3 min in 70% ethanol and for 20 min in sterilization solution while shaking at 50 rpm, followed by five washes with previously autoclaved water.
2. Excise immature embryos approximately 2 mm in size and in developmental stage 3, as suggested by Zimny and Lörz (27), and place on callus induction medium with the coleoptile in contact with the medium. Culture 40 to 50 explants per 90-mm Petri dish in the dark at 25°C for 5 d.
3. Grow *A. tumefaciens* strain AGL0 harboring vector pJFnpII 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 OD₆₆₀ value is 2.0–2.5).
5. Centrifuge 1 mL of the bacterial culture at 13,000g for 5 min, and discard the supernatant.
6. Resuspend the pellet and dilute the suspension to an OD₆₆₀ = 1.5 to 2.0 in 1:1 (v/v) LB medium/CCML (without antibiotics). Incubate the culture at 28°C on an orbital shaker at 230 rpm for 2 h before cocultivation.

3.2. Inoculation, Cocultivation, Selection, and Regeneration of Transgenic Plants

1. For osmotic treatment place 25 to 50 precultured immature embryos (from **Step 2** of **Subheading 3.1.**) per 39 mm well of a 6X macroplate and suspend in 5 mL OTM medium per well for 4 to 6 h at 80 rpm in the dark.
2. Remove OTM medium with a pipet and inoculate 25 to 50 precultured immature embryos per well with approximately 300 μ L *Agrobacterium* suspension (from **Step 6** of **Subheading 3.1.**), vacuum-treat at 500 to 800 mbar for 1 min, and keep in the laminar flow hood for 10 min.
3. Remove *Agrobacterium* suspension with a pipet and rinse explants gently two times with 5 mL CCML medium to remove excess *Agrobacterium*, followed by a coculture in 5 mL CCML medium overnight at 22°C at 80 rpm in the dark (see **Note 8**).
4. After 14 to 16 h of cocultivation in CCML medium, rinse explants thoroughly (at least five times) in CCML medium, and blot the explants dry with sterile filter paper, followed by an immediate transfer to CCMS medium (25 explants/90-mm Petri dish). With the coleoptile in contact with the medium, culture for 2 d at 22°C in the dark (see **Note 8**).
5. Transfer explants to CIM to promote callus and suppress *Agrobacterium* growth after the cocultivation. Maintain cultures in the dark for 2 wk at 25°C (see **Note 9**).
6. Transfer calli to 90-mm Petri dishes with SRM I and culture at a 16-h photoperiod, 60 μ E/m²/s illumination at 25°C for shoot regeneration for 3 wk (see **Note 9**).
7. Transfer five regenerating calli to a 60 $\times$ 100 $\times$ 100-mm (L $\times$ W $\times$ H) or similar container with 60 mL SRM II and culture at a 16-h photoperiod at 25°C with 60 μ E/m²/s illumination for shoot elongation and root formation of transgenic shoots within 3 wk (see **Notes 9** and **10**).
8. It takes approximately 4 mo from transplanting until harvest of mature seeds. Rooted transgenic plantlets are transferred to soil after carefully washing off medium from the roots. Topsoil, peat, and sand are mixed in a 3:1:1 ratio for plant growth.
9. To support acclimation, plantlets are covered with a Magenta box (Sigma) for the first 4 d following transfer to soil. Plants are grown in the growth chamber at 12°C to 16°C during the night and 16°C to 20°C during the day with a 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.
10. Plants are fertilized biweekly with Peters fertilizer (20:20:20 with micronutrients), following the manufacturer's recommendations. A light intensity of 400 μ E/m²/s at plant height is maintained with metal halide lights until the end of tillering. After tillering, sodium vapor lights provide the same light intensity.
11. As soon as immature inflorescences emerge and before pollination, inflorescences are covered with cellophane bags, and pollination within the bags is enhanced with daily agitation of the bags. Under these conditions more than 90% of the transgenic lines were self-fertile, and seed set varied between 30 and 80%.

12. Regenerated plants and their progeny can be assessed for expression of the *nptII* transgene by a commercially available ELISA kit (Agdia, Elkhart, IN) (see **Note 11**). Southern blot analyses must be performed to confirm stable integration of the transgenes, and Northern blot, Western blot, or ELISA analyses are used to confirm expression of the transgenes (see **Note 12**).

4. Notes

1. Tissue culture response is highly dependent on donor plant quality and the individual genotype. Therefore it is important to avoid stresses including drought, temperatures above 25°C, pests, diseases, and pesticides. To identify alternative responsive genotypes, a large number of inbred lines (>10) should be screened. We were able to use a similar protocol successfully for barley transformation (cv. Golden Promise).
2. Developmental stage 3 is typically reached 10 d after pollination. However, owing to differences in environmental conditions and genotypes, it is important to monitor the developmental stage rather than a specific timeframe.
3. Cocultivation in a medium rich in auxins induces cell division and callus proliferation and maintains tissues in an undifferentiated state, which might enhance transformation competence.
4. It was not investigated whether the supplementation of CCML and/or CCMS medium with acetosyringone, used prior and/or during cocultivation, influences transformation efficiency in rye.
5. Gelling agents may cause precipitation of paromomycin, rendering it inactive as a selective agent. Agarose (type I, Sigma) does not cause precipitation of paromomycin.
6. The binary plasmid used (pJFnptII; **23**) has a pPZP111 vector backbone (**26**) with bacterial resistance to kanamycin. *A. tumefaciens* strain AGL0 has chromosomal resistance to rifampicin (**24**).
7. 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* culture can be grown overnight (as a subculture of the culture initiated in 100 mL LB medium as described in **Step 3** of **Subheading 3.1.**). After mixing 1:1 with an autoclaved glycerol solution (30%) the aliquoted suspension can be stored for several months at -80°C.
8. Rye tissue cultures are very sensitive to *Agrobacterium* overgrowth. The described liquid cocultivation protocol and subsequent thorough rinsing of the explants and the use of timentin reduces the potential of *Agrobacterium* overgrowth.
9. Rye tissue cultures lose their plant regeneration capacity quickly during extended tissue culture and in the presence of selective agents. Therefore this optimized protocol has a very short tissue culture period, avoids selection during callus culture, and instead selects only during the plant regeneration phase.
10. Paromomycin, compared with other selective agents, is the superior in suppressing elongation of nontransgenic shoots. Owing to the short selection period, 30 to 80% of the regenerated plants have been identified as nontransgenic escapes.

11. The removal of the selectable marker genes from transgenic elite events can be achieved by *Agrobacterium*-mediated cotransformation of unlinked T-DNA's followed by segregation analysis in sexual progenies (11,12). We also demonstrated recently that selectable marker-free transgenic rye plants can be generated without the use of selectable marker genes by PCR screening. For this approach the timing between gene transfer and initiation of regeneration is most critical (29).
12. The majority of plants generated with this protocol showed a simple transgene integration pattern with a single transgene copy, defined T-DNA borders, and exclusion of vector DNA, which supports the stability of transgene expression in subsequent generations (16).

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Sorghum (*Sorghum bicolor* L.)

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Summary

This chapter describes a stepwise protocol for *Agrobacterium*-mediated sorghum genetic transformation. Immature embryos from sorghum plants were used as the target explants. The *Agrobacterium* strain LBA4404, carrying a “super-binary” vector, was used in this protocol. *Agrobacterium* co-transformation vectors, one T-DNA containing the selectable marker gene and another T-DNA containing the trait gene(s), were also introduced in sorghum transformation for eliminating the selectable marker gene in the resulting transgenic plants. This chapter provides recommendations for analysis of the transgenic plants to confirm T-DNA integration into the sorghum genome and segregation of the selectable marker gene from the trait gene(s).

Key Words: *Agrobacterium tumefaciens*; sorghum; transformation; co-transformation; plant transformation; transgenic sorghum; transgenic plants; transgene segregation, super-binary vector.

1. Introduction

Genetic manipulation of sorghum (*Sorghum bicolor* L.) through transformation has been studied for years. However, sorghum has been categorized as one of the most difficult plant species for tissue culture and transformation (**1**). Sorghum tissue culture and plant regeneration have been successful with certain explants (**2–6**). Exploration on sorghum transformation began more than a decade ago, and much less success has been achieved compared with other crops. In recent years, significant progress has been made in *Agrobacterium*-mediated crop transformation including rice (**7–9**), maize (**10–13**), wheat (**14**), barley (**15**), and sorghum (**16,17**). *Agrobacterium*-mediated sorghum transformation was reported by inoculating meristem tissue (**18**) or the shoot apex (**19**). However, acceptance of those results is hindered by the absence of solid evidence to support stable T-DNA integration into sorghum genome. The first

report of successful sorghum transformation mediated by *Agrobacterium* with solid supporting data was published in 2000 (16).

A public sorghum cultivar, P898012, has demonstrated good transformation capability by both biolistic bombardment (20,21) and *Agrobacterium* (16). This line has also been well adapted in agriculture in Niger and Sudan because it has both preflowering and postflowering drought resistance.

In this chapter, a stepwise protocol for *Agrobacterium*-mediated sorghum immature embryo transformation is provided. The transformation process includes preparation of *Agrobacterium*, preparation of sorghum immature embryos, *Agrobacterium* infection of sorghum immature embryos, co-cultivation of sorghum immature embryos with *Agrobacterium*, resting, selection of transformed callus, plant regeneration, growth of transgenic plants, confirmation of stable transformation, and segregation of the selectable marker gene from the trait gene(s).

In our system, T_0 plants are the directly regenerated transgenic plants from transgenic calli, and T_1 is the first progeny of the T_0 plants. A transgenic event is defined as one or more fertile T_0 plants derived from a single embryo showing T-DNA integration into the sorghum genome by Southern blotting. Transformation frequency is defined as the number of transgenic events produced from 100 infected immature embryos. With the protocol provided here, about 2% transformation efficiency can be achieved in the sorghum line P898012 (see Note 1).

The elimination of the selectable marker gene from transgenic plants, especially in commercial products, has received significant attention. The persistence of the marker gene in the products is undesirable or unacceptable. This is more important in sorghum since crop sorghum can spontaneously hybridize with johnsongrass (*Sorghum halepense* L.) under native conditions (22–25). This protocol introduces the *Agrobacterium* co-transformation vector for sorghum transformation to segregate the selectable marker gene from trait genes for generating transgenic sorghum containing trait gene(s) only as the final product.

2. Materials

2.1. Plant Material

Sorghum seeds of P898012 were provided by Dr. John Axtell (Department of Agronomy, Purdue University, West Lafayette, IN 47907).

2.2. *Agrobacterium* Strain and Vector

1. Use *Agrobacterium tumefaciens* LBA4404 and a super-binary vector constructed with pSB1 and pSB11 (26,27) for sorghum transformation. The super-binary vector contains a selectable marker gene, *bar* (28) (see Note 2) within the two bor-



Fig. 1. Maps of the T-DNA regions of *Agrobacterium* vector PHI11262 and PHI11264. UBI, maize ubiquitin promoter and maize ubiquitin intron 1; B, bar; t, *pin*-II terminator; RB, T-DNA right border; LB, T-DNA left border.



Fig. 2. Map of the T-DNA regions of an *Agrobacterium* co-transformation vector. GZP, 27-kD gamma zein promoter; H, HT-12; T, 27-kD gamma zein terminator; UBI, maize ubiquitin promoter; INT, maize ubiquitin intron1; B, bar; t, *pin*-II terminator; RB, T-DNA right border; LB, T-DNA left border.

ders of the T-DNA. Other marker genes, such as intron green fluorescent protein (GFP) (29) or intron β -glucuronidase (GUS) (30–32), may be used for optimal event recovery. As an example, a DNA fragment containing the maize ubiquitin (Ubi) promoter (33) driving a *bar* gene is inserted between the T-DNA borders and the *pin*-II (34) terminator is ligated downstream of the *bar* coding sequence in pSB11 to construct the transformation vector PHP11264. In another example, vector PHP11262, the *bar* gene is replaced with an intron-GUS gene. The T-DNA regions of these two vectors are shown in Fig. 1.

2. The design of the co-transformation vector is based on the work of Komari et al. (27). There are two T-DNAs, one containing the selectable marker gene and another containing the trait gene(s) on the same super-binary vector. As an example, three copies of the high-lysine gene HT-12 (35), under the control of the 27-kDa maize gamma zein promoter and terminator (36), are linked together within one T-DNA border, and a *bar* coding sequence controlled by the maize ubiquitin promoter and *pin*-II terminator is assembled within another T-DNA border (Fig. 2).

2.3. Media

2.3.1. Media for *Agrobacterium* Preparation

1. Minimal AB: 50 mL/L stock A, 50 mL/L stock B, 5 g/L glucose, 9 g/L Phytagar. For the *Agrobacterium* strain used in this protocol, add 1 mL of 50 mg/mL stock solution of spectinomycin per liter of medium after autoclaving.

- a. Stock A: 60 g/L K_2HPO_4 , 20 g/L NaH_2PO_4 , pH 7.0.
- b. Stock B: 20 g/L NH_4Cl , 6 g/L $MgSO_4 \cdot 7H_2O$, 3 g/L KCl , 0.2 g/L $CaCl_2$, 0.5 g/L $FeSO_4 \cdot H_2O$.
2. YP medium: 5 g/L yeast extract, 10 g/L peptone, 5 g/L $NaCl$, 15 g/L Bacto-agar; autoclave. For the *Agrobacterium* strain used in this protocol, add 1 mL of 50 mg/mL stock solution of spectinomycin per liter of medium before pouring into 100 × 15-mm Petri dishes.

2.3.2. Stock Solutions for Sorghum Transformation

1. 0.1 M Acetosyringone stock: dissolve 490 mg 3',5'-dimethoxy-4'-hydroxy-acetophenone (Aldrich, cat. no. D13,440-6) in 25 mL DMSO, filter-sterilize, and store at $-20^\circ C$ in 1-mL aliquots.
2. Bialaphos stock (see **Note 3**): to prepare from herbicide:
 - a. Obtain Herbiace® herbicide from Meiji Seika K.K., Japan, containing 20% active ingredient, bialaphos.
 - b. Mix 20 mL Herbiace with 80 mL DI water.
 - c. Prepare a BAKERBOND *spe* column (VWR, cat. no. JT7020-13): add 1.5 to 2 mL absolute methanol to each of 12 columns held in a column processor and collect samples with Falcon 15-mL tubes (VWR, cat. no. 21008-935) and verify that columns are empty of methanol (no drips); then flush each column with 2 to 2.5 mL DI water, remove Falcon tubes, and replace with fresh tubes.
 - d. Add 2 mL of Herbiace dilution to each of the conditioned columns; do not apply vacuum.
 - e. When the green front of the herbicide reaches the fritted disk, turn stopcock off.
 - f. Combine elutes from columns; the bialaphos fraction in the tubes should be straw colored.
 - g. Determine bialaphos concentration: sample 5 μL of elute and dilute with 1995 μL DI water (1:400 dilution), measure OD at 205 and 280 nm, and compute bialaphos concentration with the formula $E = 27 + 120 (OD_{280}/OD_{205})$ in mg/mL; multiply by 400 for the original concentration.
 - h. Store bialaphos at $-20^\circ C$.
 - i. Dilute bialaphos to 1 mg/mL for use in media and store in refrigerator no longer than 2 mo.
 - j. Filter-sterilize before adding to media.
3. MS vitamins stock: dissolve 10 mg nicotinic acid, 10 mg pyridoxine HCl, 2 mg thiamine HCl, and 40 mg glycine in 100 mL of DI water; filter-sterilize and store at $4^\circ C$.
4. Antibiotic and phytohormone stock solutions.
 - a. 50 mg/mL Spectinomycin: dissolve 500 mg spectinomycin (Sigma, cat. no. S-9007) in 50 mL of DI water and add DI water to 100 mL. Filter-sterilize and store at $-20^\circ C$.
 - b. 100 mg/mL Carbenicillin: dissolve 10 g carbenicillin (ICN, cat. no. 195092) in 50 mL of DI water and add DI water to 100 mL. Filter-sterilize and store at $4^\circ C$.

- c. 1 mg/mL Nicotinic acid: dissolve 100 mg of nicotinic acid (Sigma, cat. no. N-4126) in 50 mL of DI water, add DI water to 100 mL, and store at 4°C.
- d. 1 mg/mL Pyridoxine HCl: dissolve 100 mg pyridoxine HCl (Sigma, cat. no. P-9755) in 50 mL of DI water, add DI water to 100 mL, and store at 4°C.
- e. 0.4 mg/mL Thiamine HCl: dissolve 40 mg thiamine HCl (Sigma, cat. no. T-4625) in 50 mL of DI water, add DI water to 100 mL, and store at 4°C.
- f. 0.5 mg/mL 2,4-D: dissolve 50 mg 2,4-D (Sigma, cat. no. D-4517) in 2.5 mL of 0.2 *N* KOH, sonicate until completely dissolved, add DI water to 100 mL, and store at 4°C.
- g. 10 mg/mL Ascorbic acid: dissolve 1 g ascorbic acid (Sigma, cat. no. A-4544) in 50 mL of DI water, add DI water to 100 mL, and store at 4°C.
- h. 0.5 mg/mL Kinetin: dissolve 50 mg kinetin (Sigma, cat. no. K-1885) in 50 mL of DI water, add DI water to 100 mL, and store at 4°C.
- i. 1 mg/mL Indoleacetic acid (IAA): dissolve IAA (Sigma, cat. no. I-2886) in 50 mL of DI water, add DI water to 100 mL, and store at 4°C.
- j. 0.1 mM Abscissic acid (ABA): dissolve 3 mg ABA (Sigma, cat. no. A-1049) in a few drops of 0.1 *N* KOH, add DI water to 100 mL, and store at 4°C.
- k. 1 mg/mL Thidiazuron (1-phenyl-3-[1,2,3-thiadiazol-5-yl]-urea): dissolve 5 mg thidiazuron (Sigma, cat. no. P-6186) in 1 mL of 1 *N* KOH and store at -20°C. Add DI water to 1 mg/mL before use.
- m. 1 mg/mL 1-Naphthaleneacetic acid (NAA): dissolve 100 mg NAA (Sigma, cat. no. N-0640) in 50 mL of DI water, add DI water to 100 mL, and store at 4°C.
- n. 1 mg/mL Indole-3 butyric acid (IBA): dissolve 100 mg IBA (Sigma, cat. no. I-5386) in 50 mL of DI water, add DI water to 100 mL, and store at 4°C.

2.3.3. Media for *Sorghum* Transformation

1. PHI-I: 4.3 g/L MS salts (GIBCO BRL, cat. no. 11117-874), 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine HCl, 1 mg/L thiamine HCl, 0.1 g/L myoinositol, 1 g/L vitamin assay casamino acids (DIFCO, cat. no. 0230-01-1), 1.5 mg/L 2,4-D, 68.5 g/L sucrose, 36 g/L glucose, pH 5.2; autoclave. Add 100 μ M acetosyringone before using.
2. PHI-T: add PHI-I with reducing sucrose to 20 g/L and glucose to 10 g/L, increasing 2,4-D to 2 mg/L, adding 0.5 g/L 2-[*N*-morpholino]ethanesulfonic acid (MES; Sigma, cat. no. M-8250) buffer, 0.7 g/L L-proline, 10 mg/L ascorbic acid, pH 5.8, 8 g/L agar; autoclave. Add 100 μ M acetosyringone before pouring medium to 100 $\times$ 25-mm Petri dishes.
3. PHI-U: PHI-T with 1.5 mg/L 2,4-D, 100 mg/L carbenicillin, 5 mg/L PPT, without glucose and acetosyringone.
4. PHI-V: PHI-U with 10 mg/L PPT.
5. PHI-W: PHI-U with 0.5 mg/L kinetin.
6. PHI-X: 4.3 g/L MS salts, 0.1 g/L myoinositol, 5.0 mL MS vitamin stock solution, 0.5 mg/L zeatin, 700 mg/L proline, 60 g/L sucrose, 1 mg/L IAA, 0.1 μ M ABA, 0.1 mg/L thidiazuron, 5 mg/L PPT, 100 mg/L carbenicillin, 8 g/L agar, pH 5.6.

7. PHI-FA: 2.15 g/L MS, 0.05 g/L myoinositol, 2.5 mL MS vitamin stock solution, 20 g/L sucrose, 0.5 mg/L NAA, 0.5 mg/L IBA, 7 g/L agar, pH 5.6.
8. PHI-Z: PHI-FA without NAA and IBA.

3. Methods

3.1. Assessment of a Sorghum Genotype for *Agrobacterium* Transformation

A sorghum line with good tissue culture response and plant regeneration capability is not necessary a good candidate for *Agrobacterium*-mediated transformation. To evaluate a sorghum genotype with good potential for *Agrobacterium* transformation, usually two major aspects should be considered: good capability to receive T-DNA delivery and minimal damage caused by *Agrobacterium*.

1. Infect a group of immature embryos (20–50) with *Agrobacterium* carrying a visual marker gene, such as intron-GFP, and co-cultivate these embryos for 3 d with the protocol described in **Subheadings 3.2., 3.3., and 3.4.**
2. Observe the expression of the visible marker gene in these embryos and give scores as high, intermediate, low, or none.
3. Continue to culture these embryos on resting medium (*see Subheading 3.5.*) for 2 to 4 wk to evaluate callus initiation frequency and compare with the control cultures (embryos from the same panicles cultured on resting medium directly without *Agrobacterium* infection). Meanwhile, observe the expression of the visual marker gene again and verify the association of the expression of the marker gene with freshly initiated callus.
4. Usually a sorghum genotype with intermediate or high expression of the visual marker gene, expression of the marker gene associated with some of the callus, and 50% or higher frequency of callus initiation compared with the controlled cultures (defined as 100%) is recommended as the target line for *Agrobacterium*-mediated transformation.

3.2. Preparation of *Agrobacterium*

1. Master plate: store *Agrobacterium* strains as glycerol stocks at -70°C . Using standard microbiological technique, streak a loop full of bacteria to produce single colonies on minimal AB medium (containing appropriate antibiotics) in a $100 \times 15\text{-mm}$ Petri dish and incubate the plate, inverted, at 28°C in the dark for 2 to 3 d. Bacteria on a master plate are usable for up to 4 wk if the plates are sealed with Parafilm and stored in the cold (4°C).
2. Working plate: pick two to three colonies from a master plate and streak a fresh plate of YP medium (containing appropriate antibiotics). Incubate bacterial plate inverted, at 28°C , in the dark, for 1 to 2 d (*see Note 4*).
3. Suspension for immature embryo infection:
 - a. Add acetosyringone to 20 mL of PHI-I medium (pre-warmed to room temperature) in a 100-mL tube to make PHI-I with $100\text{ }\mu\text{M}$ acetosyringone.

- b. Scrape two to three loopfuls of bacteria off the working plate with a sterile bacteria loop and place in PHI-I with 100 μM acetosyringone.
- c. Vigorously vortex the tube to break the clumps. A uniform suspension culture can be determined by visual inspection.
- d. Take 1 mL of *Agrobacterium* suspension to measure optical density at 550 nm. Dilute the suspension with PHI-I plus 100 μM acetosyringone to 10^9 cfu/mL (OD at 0.7) (see **Note 5**).

3.3. Preparation of Immature Embryos

1. Healthy sorghum plants grown under greenhouse, growth chamber or field conditions are chosen for transformation. Harvest immature panicles 9 to 12 d post pollination depending on the growing conditions. The size of the immature zygotic embryos used in transformation ranges from 1.0 to 1.5 mm in length.
2. Take off immature kernels from the panicles and sterilize them with 50% bleach and 0.1% Tween-20 for 30 min with the bench vacuum; then rinse the kernels with sterile water three times (2–3 min each rinse). Keep the kernels in sterile water before isolating the embryos.
3. Aseptically dissect embryos from each sterilized sorghum kernel and place in a 2-mL microtube containing 2 mL PHI-I with 100 μM acetosyringone. Usually, place about 100 embryos per tube, but this can vary.

3.4. *Agrobacterium* Infection and Cocultivation of Embryos

1. Once all embryos are dissected, remove PHI-I liquid medium from the tube with a 1-mL micropipetor and add 1 mL *Agrobacterium* suspension.
2. Gently invert the tube a few times to mix well.
3. Incubate for 5 min at room temperature.
4. Remove *Agrobacterium* suspension from the tube with a 1-mL micropipetor.
5. Scrape the embryos from the tube using a sterile spatula.
6. Transfer immature embryos to a plate of PHI-T (see **Notes 6** and **7**) medium in a 100 $\times$ 15-mm Petri dish. Orient the embryos with embryonic axis down on the surface of the medium.
7. Incubate these embryos at 25°C (see **Note 8**) in the dark for 3 d.

3.5. Resting

Transfer embryos to PHI-T plus 100 mg/L carbenicillin but minus acetosyringone with the same embryo orientation and incubate at 28°C in the dark for 4 d.

3.6. Selection of Putative Transgenic Events

1. Transfer embryos to PHI-U medium and incubate at 28°C in the dark for 2 wk.
2. Transfer embryos to PHI-V medium and incubate at 28°C in the dark for the remainder of the selection process. The subculture interval is typically 2 wk; however, if the sorghum tissue produced more phenolic pigment, the subculture

interval can be reduced to 5 to 7 d (5). The whole process of callus selection usually takes about 10 wk depending on the genotypes of the sorghum line. The bottom line is getting enough high-quality freshly growing callus for plant generation.

3.7. Regeneration of T_0 Plants

1. Transfer the herbicide-resistant callus to PHI-W medium and incubate at 28°C in the dark for 2 to 3 wk to increase the quantity of the putative transformed callus and to develop somatic embryos.
2. Transfer these calli on PHI-X medium and incubate at 28°C in the dark for approximately 2 to 3 wk to develop shoots.
3. When shoots start to appear, move these cultures to a lighting culture room under conditions of 16 h light (270 $\mu\text{E}/\text{m}^2/\text{s}$) and 8 h dark at 25°C for a week or so.
4. Transfer shoots (about 3–5 cm tall) to plastic boxes (10 $\times$ 9 $\times$ 10 cm) containing either PHI-Z medium if the shoots had good roots or PHI-FA medium if the shoots had no or poor roots. Culture these shoots under the same light and temperature conditions for 3 to 5 d. Each box contains shoots derived from a single embryo.
5. When the plantlets reach about 8 to 10 cm tall with healthy roots, transfer these plantlets to pots with Universal Mix (Strong-Lite, Seneca, IL) in the greenhouse.

3.8. Greenhouse Care of Transgenic Sorghum

Greenhouse care of transgenic T_0 plants is an important part of the sorghum transformation process. Appropriate growth conditions improve the health and fertility of T_0 plants.

1. Transfer the plantlets from the boxes into an 18-well flat and place the flat in a temperature (26°C with light and 21°C without light) and light (16 h light and 8 h dark) controlled growth chamber for a week to grow healthy root system.
2. Transfer the plants to 1-gallon pots, one plant per pot, and grow in the greenhouse with 16 h light and 8 h dark at 18 to 30°C. T_0 sorghum plants usually grow about 3.5 mo in the greenhouse from transplant to harvest.
3. Sorghum is a self-pollinated species, and in open pollination condition, the out-cross rate ranges from 0.1 to 30%. To avoid any cross-pollination between the T_0 plants derived from different transgenic events, cover the panicles with pollination bags prior to flowering.
4. Harvest and dry the panicles and take off seeds from the panicles. Usually 50 to 200 kernels per panicle can be obtained.

3.9. Confirmation of Transformation

1. Analyze the putative transgenic events to confirm T-DNA integration into the sorghum genome. The choice of specific analytical methods performed on any transgenic event is dependent on the transgene. In general, all events are assayed for the presence of the transgene by polymerase chain reaction (PCR) or/and Southern blot analysis. The expression of the transgene(s) can also be assayed.

For example, in the events produced with the *gus* gene, tissues such as callus, leaf, roots, pollen, seeds, and so on are stained with a GUS histochemical assay reagent. If the events are transformed with a *bar* gene, the transgenic plants can be painted with bialaphos herbicide (1% v/v; Liberty). The subsequent lack of herbicide-injury lesion indicates the presence and expression of the *bar* transgene in these transgenic plants. The Southern blot method is also used to determine the pattern of the integrated T-DNA, such as copy number, truncation, rearrangement, and so on.

2. To demonstrate stable transformation, confirmation of the presence of the transgene(s) in the sorghum genome should be at least in two generations, T₀ and T₁, by the Southern blot method.
3. With *Agrobacterium*-mediated transformation, one of the important issues is the integration of the T-DNA vector backbone sequence into the sorghum genome (11,37,38). To elucidate the integration of the backbone sequence, Southern blot or PCR analyses against the vector backbone sequence should be performed.

3.10. Segregation of Selective Marker from Trait Gene(s)

1. Germinate about 50 to 100 T₁ seedlings from each event in flats and confirm the presence or absence of the transgenes in individual T₁ seedlings by either PCR or Southern blot or by expression of these transgenes (see Note 9).
2. Determine the events that demonstrate segregation of the transgenes cloned on two T-DNAs based on the analytical results of these 50 to 100 T₁ seedlings in each event.
3. Transplant the T₁ seedlings that contain the trait gene(s) only and do not contain the selectable marker gene and/or other unnecessary DNA fragment(s), as well as vector backbone, into pots. Grow these plants to maturity and harvest the seeds.

4. Notes

1. This protocol can be used for other sorghum genotypes, such as PHI391 (16).
2. The *pat* gene (39) can also be used as the selectable marker for sorghum transformation.
3. Herbiace can be purchased in the agricultural supply market in Japan.
4. *Agrobacterium* suspension for infection can also be prepared in liquid shake culture. One day prior to transformation, about 30 mL of minimal AB medium in a 30-mL baffle flask containing 50 µg/mL spectinomycin is inoculated with a 1/8 loop-full of *Agrobacterium* from a 1- to 2-d-old working plate. The *Agrobacterium* is grown at 28°C at 200 rpm in the dark overnight (about 14 h). In mid-log phase, the *Agrobacterium* cells are harvested and resuspended at 10⁹ cfu/mL in PHI-I medium + 100 µM acetosyringone using standard microbial techniques and standard curves.
5. If 10⁹ cfu/mL of *Agrobacterium* suspension causes serious damage of immature embryos of a sorghum line, lower concentrations of *Agrobacterium* suspension should be tested.

6. If sorghum tissues produce more phenolic pigment, 1% polyvinylpyrrolidone (PVPP) can be added to co-cultivation medium PHI-T to protect sorghum tissues (16). In addition, PVPP can be added to the resting medium (PHI-T plus 100 mg/L carbenicillin and minus acetosyringone) and the selection medium (PHI-U and PHI-V) to protect callus growth.
7. L-cysteine can be used in the co-cultivation phase. Cocultivation medium supplemented with 100 to 400 mg/L L-cysteine may help in recovering stable transgenic events (13).
8. Lower temperature for cocultivation, such as 20 to 22°C, may be better for some sorghum lines (40).
9. PCR and herbicide Liberty® painting can be used to screen the selectable marker gene. However, final confirmation of the absence of these genes in the transgenic plants has to be done by Southern blotting. Sometimes truncated or rearranged fragments of these genes can be inserted into the sorghum genome; in many cases these fragments are not expressed well and are not detectable by PCR.

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Wheat (*Triticum aestivum* L.)

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Summary

This chapter describes a procedure for *Agrobacterium*-mediated wheat transformation. Freshly isolated immature embryos, precultured immature embryos, or embryogenic calli are inoculated with a disarmed *A. tumefaciens* strain C58 (ABI) harboring the binary vector pMON18365 containing the β -glucuronidase (GUS) gene with an intron, and a selectable marker, neomycinphosphotranferase (NPT) II gene. The inoculated explants are selected on callus induction medium with the selective agent G418 for approximately 2 wk. The resistant callus pieces that develop are then transferred onto medium with the selective agent and reduced plant growth regulators for plant regeneration and further selection. It takes approximately 2.5 to 3 mo from inoculation to the establishment of R_0 plants in soil. All the transformants should be morphologically normal and set seeds. Approximately 35% of the transgenic plants have a single copy of the transgene based on data obtained from previous experiments.

Key Words: *Agrobacterium tumefaciens*; wheat; *Triticum aestivum* L.; plant transformation; monocotyledon.

1. Introduction

Wheat is one of the most important crops in the world. As the world population increases, the demand for wheat will continue to grow. With limited natural resources, it is essential to increase the yield and quality continuously to meet the demand. Conventional breeding, which has contributed greatly to the genetic improvement of wheat, has limitations as a useful genetic resource. Genetic engineering allows introduction of foreign genes from unrelated organisms into the wheat germplasm and provides an unlimited gene pool. The success of genetic improvement through genetic engineering depends on an efficient procedure for introducing a single or a low copy number of defined foreign genes into the plant genome. *Agrobacterium*-mediated plant transformation

has advantages over direct DNA delivery techniques, such as the particle-gun method, which include a higher frequency of transformants with single or low copies of the intact transgene, increased frequency of coexpression, and enhanced stability of expression over generations (1).

During the 1980s and early 1990s, monocotyledonous species including wheat were considered to be difficult to transform via *Agrobacterium*. One transformation method was reported for rice in 1994 by Hiei et al. (2). Isida et al. (3) reported one method for *Agrobacterium*-mediated transformation of maize. In work published in 1997, an efficient *Agrobacterium*-mediated wheat transformation method was reported by our group (4). A standard binary vector, rather than a “super”-binary vector as described in the rice and maize protocols (2,3), was successfully used in wheat transformation. Multiple kinds of explant materials were used for *Agrobacterium* infection and generated transgenic plants through NPTII selection. Over the years, this method has been shown to be efficient and yields a high transformation frequency. A transformation frequency up to 4% should be expected. The frequency is defined as percent of inoculated explants producing transgenic plants, which are confirmed by Southern hybridization. Approximately 35% of the transgenic events have single gene inserts. The transformation timeframe (from inoculation to transplanting R₀ plants to soil) is approximately 2.5 to 3.0 mo. The method has also been used efficiently for producing transgenic wheat plants through glyphosate selection (5,6). One example of the method is described below.

2. Materials

2.1. Plant Materials

1. Wheat plants (CV Bobwhite, a spring wheat) growing in greenhouse or growth chamber.
2. Immature caryopses collected from the plants 12 to 14 d after anthesis.

2.2. Stock Solutions and Culture Media

2.2.1. Stock Solutions

All the stock solutions may be filter-sterilized and stored at –20°C in aliquots.

1. 2,4-Dichlorophenoxyacetic acid (2,4-D), 1 mg/mL stock solution (Phyto-technology Laboratories, Shawnee Mission, KS).
2. Picloram, 1 mg/mL stock solution. Prepared by dissolving 1 g picloram in approximately 20 mL ethanol (200 proof) and 10 drops of 5 N KOH in a small beaker by stirring. Transfer it to a larger beaker and bring the volume to 1000 mL with tissue culture water. Filter-sterilize with a 0.22-μm unit.
3. Ascorbic acid, 50 mg/mL stock solution. Prepared by dissolving the powder in tissue culture water and filter-sterilizing with a 0.22-μm unit.

4. Acetosyringone, 1 M stock solution. Prepared by dissolving the powder in dimethyl sulfoxide (DMSO). Filter sterilization is not necessary.
5. Kanamycin sulfate, 50 mg/mL stock solution. Prepared by dissolving in tissue culture water with stirring. Filter-sterilize with a 0.22- μ m unit.
6. Spectinomycin, 50 mg/mL stock solution. Prepared by dissolving it in tissue culture water with shaking or vortexing. Filter-sterilize with a 0.22- μ m unit.
7. Chloramphenicol, 25 mg/mL. Prepared by dissolving in ethanol (200 proof) with vortexing.
8. Carbenicillin, 250 mg/mL. Prepared by dissolving in tissue culture water with stirring. Filter-sterilize with a 0.22- μ m unit.
9. Glucose, 0.5 g/mL stock solution. Prepared as follows: in a large beaker, add approximately 400 mL tissue culture water and heat in a microwave for 4 min. Add 500 g glucose slowly to the water while stirring. (Reheating may be necessary to dissolve.). Bring to volume (1 L) with tissue culture water. Filter-sterilize the solution with a 0.22- μ m unit.
10. G418, 25 mg/mL stock solution. Prepared by dissolving in tissue culture water and filter-sterilizing with a 0.22- μ m unit.
11. Surfactant: Silwet (Monsanto, St. Louis, MO) or pluronic F68 (Sigma, St. Louis, MO).
12. β -Glucuronidase (GUS) histochemical buffer (X-gluc buffer): as described, for example, by Jefferson (7), as 0.5 mg/mL of X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide), 100 mM sodium phosphate buffer, pH 7.0, 0.5 mM ferrocyanide, 0.5 mM ferricyanide, 0.5% (v/v) Triton X-100, 1 mM EDTA.

2.2.2. Media for Callus Induction

1. CM4C medium: 1X MS basal salts and vitamins (Phytotechnology Laboratories) (8), 0.5 g/L glutamine, 0.1 g/L casein hydrolysate, 0.75 g/L magnesium chloride, 40 g/L maltose, 0.5 mg/L 2,4-D, 2.2 mg/L picloram, 1.95 g/L 2-(*N*-morpholino)-ethanesulfonic acid (MES), pH 5.8, solidified by 2 g/L Gelrite. Autoclave and add 100 mg/L ascorbic acid to cooled media. Dispense into Petri dishes (25 $\times$ 100 mm), approx 50 mL per dish.

2.2.3. Media for Growing Agrobacterium, Inoculation, and Cocultivation

1. Luria-Bertani (LB) medium: 1% (v/v) tryptone, 0.5% (v/v) yeast extract, and 1% (v/v) NaCl, pH 7.0. Autoclave.
2. LB medium supplemented with 50 mg/L each of kanamycin, streptomycin, and spectinomycin, 25 mg/L chloramphenicol, and 200 μ M acetosyringone. Add the supplements just prior to use.
3. Semisolid LB medium supplemented with 50 mg/L each of kanamycin, streptomycin, and spectinomycin, 25 mg/L chloramphenicol, solidified with 15 g/L Agar. Add the supplements after autoclaving.
4. Inoculation medium: 1/10 strength CM4C medium: same as CM4C, but only 1/10 of the MS salts and vitamins are used.
5. Cocultivation medium: Liquid and semisolid 1/10 strength CM4C medium supplemented with 10 g/L glucose and 200 μ M acetosyringone. Add the supple-

ments after autoclaving. Dispense the semisolid medium into Petri dishes (25 × 100 mm), approx 50 mL per dish.

2.2.4. Media for Selection, Callus Induction, and Regeneration (see **Note 1**)

1. CM4C medium supplemented with 250 mg/L carbenicillin (added after autoclaving). Dispense into Petri dishes (25 × 100 mm), approximately 50 mL per dish. This is also called delay medium.
2. Semisolid CM4C medium supplemented with 250 mg/L carbenicillin and 25 mg/L G418 (added after autoclaving). Dispense into Petri dishes (25 × 100 mm), approx 50 mL per dish.
3. MMS0.2C medium: 1X MS salts and vitamins, 1.95 g/L MES, 0.2 mg/L 2,4-D, and 40 g/L maltose, pH 5.6, solidified by 2 g/L Gelrite. Autoclave and add 100 mg/L ascorbic acid.
4. MMS0.2C medium supplemented with 250 mg/L carbenicillin and 25 mg/L G418 (added after autoclaving). Dispense into Petri dishes (25 × 100 mm), approx 50 mL per dish.
5. MMS0C: same as MMS0.2C except 2,4-D is removed.
6. MMS0C supplemented with 250 mg/L carbenicillin and 25 mg/L G418. Dispense into Petri dishes (25 × 100 mm), approx 50 mL per dish. Dispense into sterile sundae cups or similar culture vessels for shoot growth.

2.3. *Agrobacterium Strain and Transformation Vector*

1. Disarmed *Agrobacterium tumefaciens* strain C58 (ABI) harboring binary vector pMON18365 (4; Monsanto) was used for the transformations in our work. The vector contains the GUS gene (*uidA*) with an intron as a screenable marker and the NPTII gene (*nptII*) as a selectable marker. Each gene is driven by an enhanced 35S (E35S) promoter.

3. Methods

3.1. *Growth of Stock Plants*

1. Grow the stock plants (Bobwhite, a spring wheat cultivar) in an environmentally controlled growth chamber or greenhouse with a photoperiod of 16-h light and 8-h dark. Set the light intensity, provided by high-intensity discharge lights (Sylvania, GTE Products, Manchester, NH), at 800 $\mu\text{mol}/\text{m}^2/\text{s}$ and the day/night temperature at 18/16°C. The plants grow in MetriMix 350 soil (Scotts, Marysville, OH), which is premixed with Osmocote fertilizer 14-14-14 and 15-9-12 (Scotts) at approximately 3.3 kg/m³ soil each.

3.2. *Preparation of Explants for Inoculation*

1. Label each wheat spike at anthesis.
2. Collect immature caryopses 14 d after anthesis and surface-sterilize with 70% ethanol for 5 min and 20% Clorox for 15 min; then wash with sterile distilled water three times.

3. Dissect immature embryos aseptically (see **Note 2**) and place on semisolid CM4C medium with the scutellum side facing upward (see **Note 3**).
4. Freshly isolated immature embryos: use the immature embryos for inoculation immediately after isolation.
5. Precultured immature embryos: inoculate the immature embryos after being cultured for 1 to 6 d in a dark incubator or culture room with temperature set at 24 to 26°C.
6. Embryogenic callus: culture the embryos on the medium for 10 to 25 d to induce callus formation. Use the intact callus pieces without breaking them apart, or select only the embryogenic callus sectors from each piece and separate them into small pieces (approx 2 mm) for inoculation.

3.3. Growth of *Agrobacterium* and Preparation of the Inoculum

1. Streak a fresh LB plate from *Agrobacterium* glycerol stock and place the plate in an incubator (28°C) for 2 to 3 d.
2. Prepare liquid medium for growing *Agrobacterium*: liquid LB medium supplemented with 50 mg/L each of kanamycin, streptomycin, and spectinomycin, 25 mg/L chloramphenicol, and 200 μ M acetosyringone. Prepare liquid LB medium as suggested in **Subheading 2.2.3.** and then add the supplements just prior to use.
3. Prepare inoculation medium: liquid 1/10 strength CM4C medium supplemented with 10 g/L glucose and 200 μ M acetosyringone. Add the supplements just prior to use.
4. One day prior to inoculation of the explants, inoculate 2 mL of liquid LB medium supplemented with the antibiotics and acetosyringone in a 15-mL test tube with a loopful of *Agrobacterium* cells from freshly streaked plate. Place the tube on a rotator for about 6 h.
5. Add 200 mL of LB medium supplemented with the antibiotics and acetosyringone and 0.2 to 1.0 mL of *Agrobacterium* culture from the 15-mL test tube in a 2-L flask.
6. Place the flask on a shaker (150 rpm) and grow overnight at 26 to 27°C to mid-log phase (optical density 1.0–1.5 at A_{660}).
7. Collect the *Agrobacterium* cells by centrifugation for 25 min at approximately 2400g and 2°C.
8. Remove the supernatant and resuspend the *Agrobacterium* cells in a small amount of the inoculation medium.
9. Adjust the *Agrobacterium* cell density to 0.5 to 1.0 at A_{660} (see **Note 4**).

3.4. Inoculation and Cocultivation

1. Transfer the explants (freshly isolated immature embryos, precultured immature embryos, or embryogenic callus pieces) to Petri dishes or other sterile containers (see **Note 5**).
2. Add prepared *Agrobacterium* inoculum to cover the explants. Set the Petri dish at room temperature for approx 3 h (see **Note 6**).

3. Remove as much inoculum as possible by vacuum or using a transfer pipet, and transfer the explants onto semisolid cocultivation medium, or a piece of filter paper saturated with liquid cocultivation medium in Petri dishes (see **Note 7**).
4. Keep the cultures at 23°C in the dark for 2 or 3 d (cocultivation phase).
5. After cocultivation, take embryo or callus samples for GUS histochemical assay. Soak the samples in the X-gluc buffer for several hours to overnight at 37°C or room temperature. GUS-positive spots (dark blue) from GUS transient expression should be visible on almost every piece (**Fig. 1**).

3.5. Selection and Callus Induction

1. Transfer the embryos and callus pieces individually from the cocultivation medium onto the delay medium (CM4C medium supplemented with 250 mg/L carbenicillin). Put the Petri dishes with the cultures, which are not sealed, in transparent plastic boxes with covers (see **Note 8**), and keep the boxes in a dark culture room or incubator with temperature set at 24 to 26°C for 2 to 5 d.
2. Transfer the embryos onto the selection medium for callus induction (CM4C medium supplemented with 250 mg/L carbenicillin and 25 mg/L G418). Put the Petri dishes with the cultures, which are not sealed, in the same boxes, and keep the cultures on this medium for 2 to 3 wk in the dark, 24 to 26°C.
3. Randomly take some callus pieces at the end of the selection for GUS histochemical assay. Some of the samples should have GUS-positive sectors, as shown in **Fig. 1**.

3.6. Selection and Plant Regeneration

1. Transfer the callus pieces onto the first regeneration medium (MMS0.2C medium supplemented with 250 mg/L carbenicillin and 25 mg/L G418) at the end of the selection and callus induction period. At the transfer, divide each callus piece derived from one immature embryo or one piece of inoculated callus into several small pieces (approx 2 mm; see **Note 9**). Keep the cultures from three to four original explants in each Petri dish.

Fig. 1. (*opposite page*) Illustration of *Agrobacterium*-mediated wheat transformation using three different kinds of explant material. Transient GUS expression was observed on almost every freshly isolated immature embryo 4 d after inoculation (**A**) and precultured immature embryo (**B**). Small GUS expression sectors were visible on immature embryo-derived callus pieces approximately 10 d after inoculation (**C**). Inoculated explants were cultured on G418-containing CM4C medium for 2 wk for callus induction and selection. At the end of this period, resistant sectors showed on some callus pieces (**D**). On the second regeneration media containing G418, putatively transformed plantlets developed (**E**). The plantlets were moved into sundae cups containing the same regeneration media for further growth and selection (**F**). Leave samples from the plantlets were assayed for GUS expression (**G**). Regenerated plants were grown in soil and fertile (**H**).

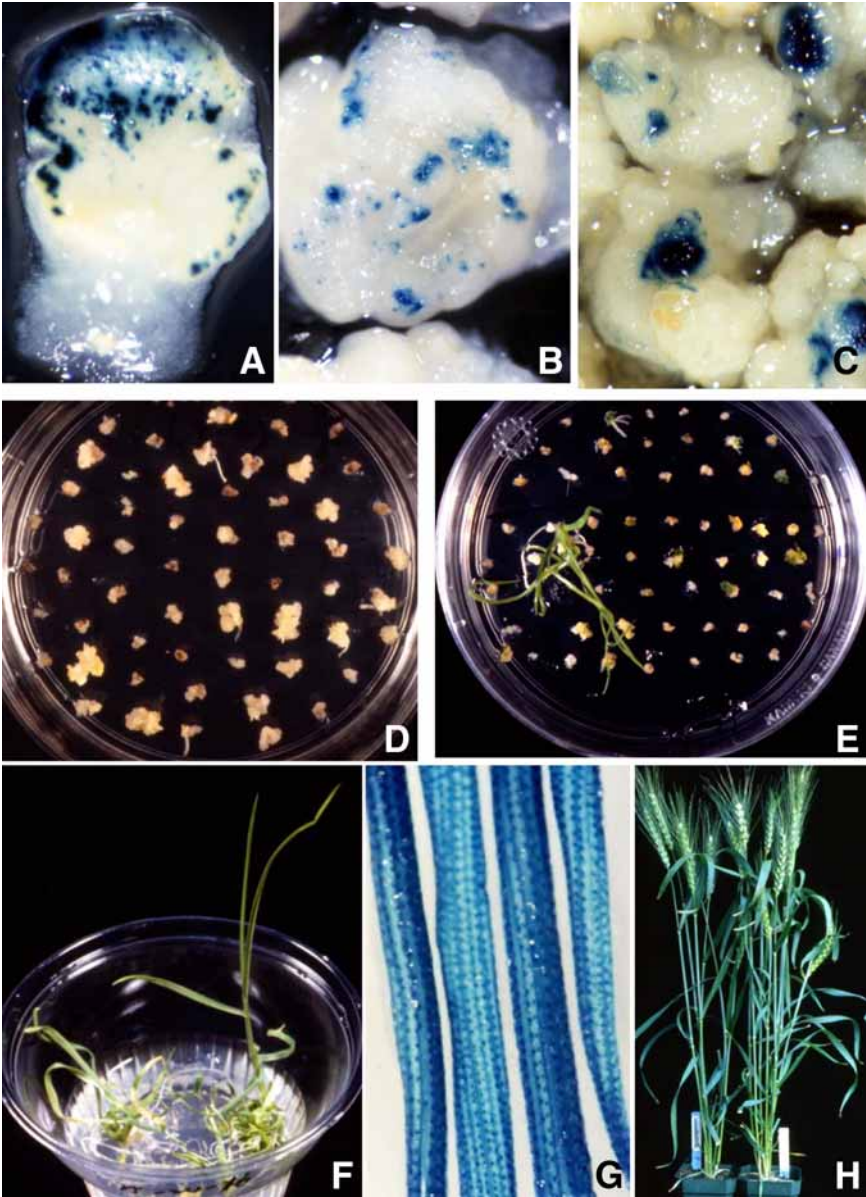


Fig. 1.

2. Keep cultures on this medium for approximately 2 wk in a culture room or incubator with a photoperiod of 16-h light (approx 50–80 $\mu\text{mol}/\text{m}^2/\text{s}$) and 8-h dark and the temperature set at 24 to 26°C.
3. Transfer developing young shoots and viable callus tissues onto the second regeneration medium (MMS0C supplemented with 250 mg/L carbenicillin and 25 mg/L G418 in Petri dishes). Make sure all the shoots and tissues from one original explant are kept together and can be separated from the cultures from other explants.
4. When the shoots develop into about 3 cm or larger plantlets, transfer them individually onto the shoot growth medium (MMS0C supplemented with 250 mg/L carbenicillin and 25 mg/L G418 in sundae cups) (**Fig. 1**). Shoots originally from one explant are treated as one clone (event).

3.7. Growth of Regenerated Plants in Soil

1. Plants that grow to reach the lid of the sundae cup and have one or more well-developed root, can be moved to soil (MetroMix 350) in 4- or 6-inch pots. The soil is premixed with fertilizer as described in **Subheading 3.1.1**.
2. Grow the plants in a growth chamber with the same environmental conditions as for the stock plants (see **Subheading 3.1**). Cover the plants with transparent plastic cups for the first 1 to 3 d in soil for acclimation. The plants are watered daily by hand or subirrigation for 1 h.
3. It takes approximately 6 wk from planting to anthesis and another 6 wk to harvest seeds. All the plants can be self-pollinated, and each should set 300 or more seeds if growing in 4-inch pots.

4. Notes

1. All the media with the antibiotics should be used within 1 wk after being made. Use of older media may result in *Agrobacterium* overgrowth on the cultures.
2. Hold the immature embryo with a pair of forceps, peel off the seed coat over the embryo, and remove the embryo with a scalpel blade.
3. The embryos or embryos with developing callus are always cultured with the scutellum side facing upward.
4. Surfactant Silwet (0.01–0.075%) or pluronic F68 (0.01–0.2%) may be added to the inoculum (resuspended *Agrobacterium* solution).
5. Embryogenic callus pieces are used as is or after being separated into small pieces (2–3 mm).
6. The inoculation time may be reduced to 30 min or less.
7. In this and later steps, when culturing the embryos or precultured immature embryos on media, orient the scutellum side of each embryo to face upward.
8. By using plastic boxes, we saved a significant amount of effort and Parafilm spent in sealing the plates.
9. All the pieces from one original explant are cultured on this medium and the second regeneration medium as one unit, so the plants regenerated from one explant are considered siblings, or from one transgenic event. Lines can be drawn on the bottom of the plate to keep the tissue from different explants separated.

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IV

INDUSTRIAL PLANTS

Canola (*Brassica napus* L.)

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Summary

High-frequency *Agrobacterium tumefaciens*-mediated transformation can be obtained in canola by optimizing the preconditioning time of the explant and cocultivation time with *A. tumefaciens*. A preconditioning time of 72 h and cocultivation of 48 h synergistically increase the transformation efficiency to 25%. In addition, the recovery of transgenic plants can be facilitated by overcoming hyperhydration, which increases the rooting frequency to 100%.

Key Words: Canola; *Agrobacterium tumefaciens*; cocultivation; preconditioning; explant; transformation; rooting.

1. Introduction

1.1. Improving *Agrobacterium tumefaciens*-Mediated Transformation

Agrobacterium-mediated transformation remains the choice of transgenic plant production in most species because of its cost effectiveness, ease in handling, and high efficiency. Constant research has improved the efficiency of *Agrobacterium*-mediated transformation. The development of new hypervirulent strains has greatly improved the efficiency of transformation and has facilitated transformation of many recalcitrant species (1). In addition, the efficiency of transformation using *Agrobacterium* may be enhanced by preconditioning the explant on callus-inducing media before the cocultivation step (2–6). When plant tissues are wounded, they secrete *vir*-inducing compounds into the surrounding medium (7). The wounded tissues are also in an active metabolic state. These actively dividing cells resulting from wound-induced cell division are more prone to transformation than nondividing cells (8). The newly synthesized cell wall is essential for the productive attachment of *Agrobacterium* preceding transformation (9). The virulence of *Agrobacterium*

can be increased by the use of acetosyringone, a phenolic compound that is now being routinely used in canola transformation (5,10).

1.2. Importance of Canola

Canola (*Brassica napus* L.) is an important oil crop, ranking third only to soybean and palm oil in global production. Economically, it is the most important member of the family *Brassicaceae* (syn. *Cruciferae*). It is a winter or spring annual crop and is amenable to growth in cooler climates. Once considered a specialty crop for Canada, it is now important globally. Many other countries including the United States, Australia, and those in Europe also grow canola. However, Canada and the United States account for most of the canola crop. It is grown mostly in western Canada and north central portions of the United States. In the year 2002, in Canada alone 9.6 million acres of canola were cultivated (11), and in the United States, 1.5 million acres were devoted to canola cultivation (12). The term “canola” was adopted by Canada apparently as an acronym of the Canadian Oilseed Association in 1979. Although canola has been commonly also known as rapeseed or oilseed rape, in the strict sense canola-quality oil must contain less than 2% erucic acid, and the solid component of the seed must contain less than 30 μmol of any one or a mixture of 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3 butenyl glucosinolate, and 2-hydroxy-4-pentenyl glucosinolate per gram of air-dried, oil-free solid. Canola quality Brassicas exist within *B. rapa* and *B. juncea*, but *B. napus* is the most widely grown canola and is the subject of this chapter.

Canola oil is widely used as a cooking oil, a salad oil, and for making margarine. Of all the edible vegetable oils widely available today, it has the lowest saturated fat content, making it appealing to health-conscious consumers. Canola oil is also used in lubricants and hydraulic fluids, especially when there is a significant risk of oil leaking to waterways or into ground water (13). It is also used in the manufacture of inks, biodegradable grease, pharmaceuticals, and cosmetics.

1.3. Need for Genetic Improvement of Canola

With the increasing demand for canola oil and the need to meet the demands of consumers, more research is being pursued to improve canola via breeding. Conventional breeding techniques are time consuming and laborious. It takes at least eight to ten generations to develop a new variety using conventional breeding. An alternative to trait improvement without conventional breeding techniques is by plant transformation, which reduces the time needed to develop a new variety. Considerable research has already been undertaken in this direction, and canola has been exploited for genetic engineering purposes (see the recent review in ref. 14). However of all the transgenic crops grown in

the world, canola represents only 7% of the total area, and there is a potential for a global increase in the cultivation of transgenic canola. Transformation could be useful to increase yields and insect and disease resistance and improve the oil quality in canola.

1.4. Improving the Efficiency of Transformation in Canola

In our work we have used hypocotyl segments as the explant of choice. Hypocotyl segment explants are highly sensitive and susceptible to necrosis. To overcome necrosis it is necessary to precondition the explants and optimize the cocultivation time with *Agrobacterium*. We have increased the transformation efficiency in canola by optimizing the preconditioning time of the explants and cocultivation time with *Agrobacterium* (5). In addition, the recovery of transgenic plants was improved by overcoming hyperhydration and increasing the rooting efficiency of the plants. The rooting efficiency was increased to 100% by manipulating the medium. With all the changes, it was possible to obtain 25% transformation efficiency. In this case we recovered 25 unique tissue-cultured shoots per 100 inoculated hypocotyl segments that were shown to be transgenic via polymerase chain reaction (PCR; progeny) and segregation analysis (5).

2. Materials

2.1. Seed Material

Brassica napus L. cv. Westar seeds, a Canadian spring-type variety is used.

2.2. *Agrobacterium tumefaciens* Strain and the Plasmid

Disarmed *A. tumefaciens* strain GV 3850 is used. It harbors the pBin-mGFP5-ER plasmid (courtesy of Jim Haseloff), which contains the *mgfp5-er* (reporter) and *nptII* gene (selectable marker), which confers resistance to kanamycin. The *mgfp5-er* gene is driven by the CaMV 35S promoter, and *nptII* is under the control of the nopaline synthase (NOS) promoter (see **Note 1**). The plasmid is transformed into competent *Agrobacterium* cells by electroporation.

2.3. Media

All solid media are autoclaved at 121°C for 20 min, and 25 mL per plate is poured into 100 × 15-mm Petri dishes except in the case of seed germination medium.

1. Liquid YEP for *Agrobacterium* culture: 10 g/L yeast extract, 5 g/L NaCl, 10 g/L peptone.
2. Seed germination medium: Murashige and Skoog (MS) (15) basal medium (see **Note 2**), 20 g/L sucrose, pH 5.8, solidified with 2 g/L Gelrite (Sigma).

3. Callus induction medium: MS medium, 1 mg/L 2,4-dichlorophenoxy acetic acid (2,4-D; Sigma), 30 g/L sucrose, pH 5.8, solidified with 2 g/L Gelrite.
4. Selection medium: callus induction medium supplemented with 400 mg/L timentin (GlaxoSmithKline) (to kill *Agrobacterium*) and 200 mg/L kanamycin monosulfide (Sigma) to select for transformed cells (see **Note 3**).
5. Organogenesis medium: MS medium supplemented with 4 mg/L 6-benzyl-aminopurine (BAP) (Sigma), 2 mg/L zeatin, and 5 mg/L silver nitrate (Sigma), antibiotics as in **item 4**, and 30 g/L sucrose, solidified with 2 g/L Gelrite.
6. Shoot regeneration medium: MS medium supplemented with 3 mg/L BAP, 2 mg/L zeatin, antibiotics, 30 g/L sucrose, and 2 g/L Gelrite for shoot development.
7. Shoot elongation medium: MS medium supplemented with 0.05 mg/L BAP and 30 g/L sucrose, solidified with 3 g/L Gelrite and antibiotics as in **item 4**.
8. Rooting medium: half-strength MS medium, 10 g/L sucrose, 3 g/L Gelrite, 0.5 mg/L indole butyric acid (IBA; Sigma), and antibiotics as in **item 4**.

2.4. Other Stock Solutions, Reagents, and Supplies

1. 1 mg/5 mL BAP stock: per 100 mL total solution, take 20 mg of BAP, wet with a few mL of 0.1 M NaOH in a weigh boat, and place a few mL of 0.1 M NaOH into the beaker. Wash wetted powder into the beaker to about 50% final volume. Stir over heat to dissolve but do not boil. Place beaker at 4°C until cool and then bring up to total volume. Do not adjust pH or autoclave. Store at 4°C.
2. 1 mg/5 mL 2,4-D stock: follow the same preparation and storage conditions as for BAP stock.
3. 1 mg/5 mL IBA stock: follow the same preparation and storage conditions as for BAP stock.
4. 1 mg/5 mL Zeatin stock: follow the same preparation and storage conditions as for BAP stock.
5. 100 mg/mL Kanamycin stock: dissolve 1 g per 10 mL water and filter-sterilize using a 0.2- μ m membrane filter. Store at -20°C.
6. 10 mg/mL Rifampicin stock: 100 g per 10 mL methanol. Filter-sterilize and store at -20°C.
7. 100 mg/mL Timentin stock: dissolve 3.1 g in 3.1 mL sterile water and store at -20°C.
8. 1 mM Acetosyringone stock: dissolve 19.62 mg in 10 mL water. Filter-sterilize and store at -20°C.
9. Soil mix: composted forest products, sphagnum peat moss, perlite, ground dolomitic limestone, a wetting agent, and a water-holding polymer, (Pro-Mix BX, Premier Horticulture, Quakertown, PA).

3. Methods

3.1. Explant Preparation

All the cultures are maintained at $25 \pm 2^\circ\text{C}$ under a 16-h photoperiod using cool white daylight fluorescent lights at 40 to 60 $\mu\text{mol}/\text{m}^2/\text{s}$.

1. Seeds are surface-sterilized for 5 min with 10% commercial bleach containing sodium hypochlorite with 0.1% Tween-20 added as a surfactant. The sterilization is followed by a 1-min rinse with 95% ethanol. The seeds are then washed thoroughly (three times) with sterile distilled water. The seeds are surface-sterilized by shaking with the sterilants manually in 1.5-mL Eppendorf tubes at a rate of 50 seeds per tube.
2. The seeds are germinated in 8 × 6-cm Magenta boxes (10 seeds per box) containing 50 mL seed germination medium for 8 to 10 d. The cultures are grown at a light intensity of 40 to 60 $\mu\text{mol}/\text{m}^2/\text{s}$.
3. Hypocotyls from the 8- to 10-d-old seedlings are cut into 1-cm pieces and preconditioned for 3 d on callus induction medium (see **Note 4**). Of special importance is the complete excision of the apical meristem. Ten to 15 explants are placed in each Petri plate, which contains 25 mL of media each.

3.2. *Agrobacterium* Culture Preparation

1. A single colony of *Agrobacterium* harboring the plasmid pBin-mGFP5-ER is grown overnight in 50 mL LB medium with 50 mg/L rifampicin and 50 mg/L kanamycin.
2. The culture is shaken on a rotary shaker at 250 rpm at 28°C. The cells are grown to an $\text{OD}_{600} = 0.8$ (see **Note 5**).
3. The *Agrobacterium* culture is then pelleted by centrifugation at 5000g for 8 min and resuspended in 15 to 20 mL liquid callus induction medium to which acetosyringone (Sigma) is added to a final concentration of 0.05 mM (see **Note 6**).
4. The culture is placed on a shaker for 30 to 120 min before transformation to activate the *Agrobacterium* virulence mechanisms.

3.3. Transformation (Fig. 1)

1. The preconditioned hypocotyls are inoculated with *A. tumefaciens* for 30 min in a Petri dish. Shake the dish at intervals manually to make sure the explants are in constant contact with *Agrobacterium*, about 100 to 150 explants per 15 to 20 mL of the *Agrobacterium* culture.
2. The explants are removed carefully from the *Agrobacterium* culture (so that excess *Agrobacterium* is drained) and transferred to callus induction medium for cocultivation for 48 h (see **Note 7**). Twenty explants can be placed on each 100 × 15-mm Petri dish.
3. For selection of transformed cells, the explants are transferred to the callus induction medium with antibiotics (400 mg/L timentin and 200 mg/L kanamycin).
4. After 2 wk, the transformed organogenetic calli are transferred to organogenesis medium (**Fig. 1A**).
5. After another 2 wk, the calli are transferred to shoot induction medium. Shoots are produced after 2 to 3 wk on this medium (**Fig. 1B**).
6. The shoots (two to four per Magenta box) are then transferred to the shoot elongation medium in Magenta boxes, which contain 25 mL of the culture medium. The shoots elongate in 3 wk (see **Note 8**).

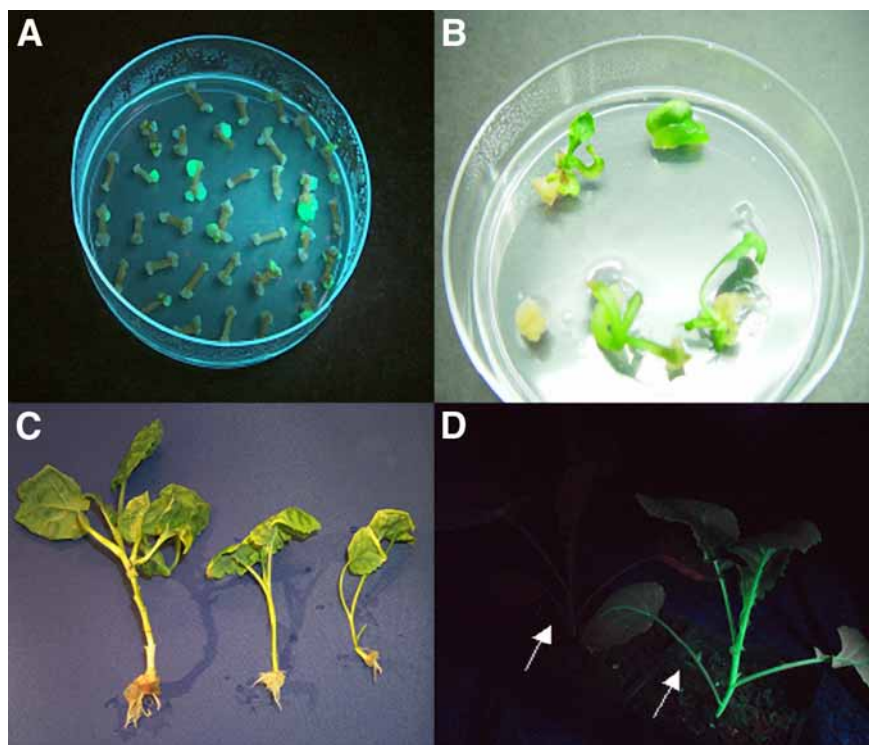


Fig. 1. Steps in genetic transformation of canola. **(A)** Hypocotyls inoculated with *Agrobacterium* showing GFP callus glowing green. **(B)** Shoots regenerating from transgenic calli. **(C)** Transgenic rooted shoots. **(D)** Transgenic canola plants in soil showing GFP expression (arrows) (in the background is a nontransgenic plant).

7. The elongated shoots are transferred to the root induction medium. Roots develop in 1 to 2 wk (see **Note 9**). Only one shoot is rooted per Magenta box, which allows the formation of well-developed roots and easy removal of the rooted shoot from the Magenta box (**Figs. 1C,D**).

3.4. Greenhouse Care

1. The rooted plants are transferred to soil mix in 1-L pots and grown in a plant growth chamber (20°C, 300 $\mu\text{mol}/\text{m}^2/\text{s}$, 16/8-h photoperiod).
2. The plantlets are covered with a plastic dome to retain humidity.
3. After 3 d, the dome is gradually removed, and the plants are transferred to 3.5-L pots and grown for seed set in the greenhouse.
4. From transplant to seed takes about 4 to 5 mo. During the first month the plants are fertilized with Peters fertilizer at a rate of 5 g/L followed by a top coat of Osmocote at 20 g per pot at mo 1.5 post transplant.
5. Plants are watered three to four times per week.

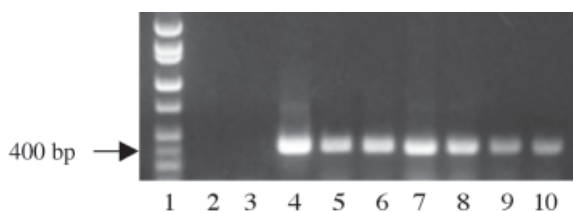


Fig. 2. Polymerase chain reaction analysis of transgenic canola with mGFP5-ER. Lane 1, DNA marker; lane 2, water; lane 3, nontransgenic control canola DNA; lane 4, contained a positive control of mGFP5-ER plasmid DNA; lanes 5–10, DNA from mGFP5-ER transgenic canola.

6. There are two applications of Marathon 1% G (Olympic Horticultural Products Mainland, PA) at a rate of 5 mL/L for insect control during the growing cycle.
7. Each plant should produce a few hundred seeds after flowers are lightly vibrated or stroked to encourage selfing.

3.5. Transgenic Plants and Progeny Analysis

3.5.1. Polymerase Chain Reaction

1. The putative transgenic plants and the T_1 transgenic plants are analyzed by PCR to confirm the presence of the transgenes.
2. DNA extraction is carried out according to [ref. 16](#).
3. The primer pairs used for green fluorescent protein (GFP) DNA amplification are 5'-ACCCAGATCATATGAAGAGG-3' and 5'-TTGGGATCTTTCGAAAGGGC-3'.
4. PCR using Promega PCR Mastermix was performed with an initial denaturation of DNA at 94°C for 5 min, then amplification by 35 cycles of 1 min at 94°C (denaturing), 1 min at 55°C (annealing), and 1 min at 72°C (extension). Amplicons are visualized by electrophoresis on a 1% agarose gel stained with ethidium bromide ([Fig. 2](#)).

3.5.2. Western Blot Analysis

See [ref. 17](#).

1. Fresh leaf tissue (0.2 g) is ground with liquid nitrogen with a hand drill-driven micropestle in a microcentrifuge tube.
2. The ground tissue is left on ice for 30 min after the addition of 0.1 *N* NaOH.
3. The homogenate is neutralized with 1 *M* Tris-HCl and centrifuged at 6200*g* for 7 min.
4. The supernatant is decanted to a fresh tube, and the total protein in each sample is determined by Bradford total protein analysis using bovine serum albumin (BSA) as a standard.
5. For the blot, 20 μ g of the sample is loaded onto a 10% polyacrylamide gel.

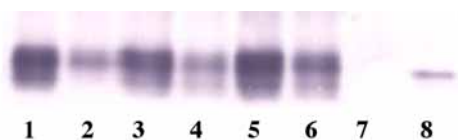


Fig. 3. Western blot analysis of GFP transgenic canola. Lanes 1–6 contained mGFP5-ER transgenic plant protein extracts; lane 7 contained protein from non-transgenic canola; lane 8 contained a GFP protein standard (27 kDa).

6. Purified GFP protein is used as a standard. The protein is transferred to a nitrocellulose membrane and immunostained, according to Pratt et al. (18).
7. The primary antibody wash was done with rabbit anti-GFP (Clontech) serum, followed by goat anti-rabbit antibody (Sigma). A rabbit antgoat alkaline phosphatase conjugate (Sigma) was used as the tertiary antibody. GFP is detected on blots by exposure to nitroblue tetrazolium/bromochloroindolyl phosphate (Fig. 3).

3.5.3. Segregation Analysis

1. Segregation analysis of the T_1 progeny is expected to result in a 3:1 Mendelian ratio if there was single-locus insertion inheritance. Approximately 20 seeds per Petri dish are surface-sterilized and sown on plates containing 200 mg/L kanamycin. Most seeds should germinate.
2. Transgenic plantlets that are kanamycin resistant should continue to grow and remain green. Nontransgenic segregants have stunted root growth, and reddish coloration and do not produce true leaves.
3. As an alternative to using a kanamycin screen, plantlets can be grown in soil or germinated in dishes and screened for GFP using a visual assay with an ultraviolet light in an otherwise darkened room (19) (Fig. 1D).

4. Notes

1. The GFP gene allows real-time monitoring of the transformed callus and shoots. GFP-positive calli and plants glow bright green when excited by UV light (19,20).
2. MS micro- and macro-salts and vitamins have been used in the medium wherever MS medium is stated.
3. Appropriate antibiotics have to be used according to the selectable marker genes on the plasmid. We use kanamycin here, since the selectable marker gene used in this work was *nptII*, which confers resistance to kanamycin, but hygromycin is also effective for selection of transformed canola tissue.
4. A preconditioning time of 72 h was found to be optimal for high transformation efficiency (5).
5. An OD of more than 1.0 decreased the transformation efficiency. Hence an OD of 0.8 to 1.0 is to be maintained for all transformation experiments
6. Acetosyringone increases the virulence of *Agrobacterium* thus increasing the transformation efficiency.

7. A cocultivation time of 48 h is optimal to obtain good transformation efficiency. With a cocultivation time of less or more than 48 h the transformation efficiency decreases significantly (5).
8. A major problem encountered in recovery of shoots from tissue culture is the hyperhydration of transformed shoots. This problem is overcome by increasing the Gelrite concentration from 2 g/L to 3 g/L in the rooting and elongation medium. By increasing the Gelrite concentration, the water availability for the shoots is reduced, thereby decreasing hyperhydration (5).
9. Rooting is very efficient using half-strength MS medium vs full-strength MS medium and reducing the sucrose concentration from 30 g/L to 10 g/L. The rooting medium we describe here gives 100% rooting in a very short time (1–2 wk). When full-strength medium is used, the plantlets elongate instead of producing roots; hence we use a low-strength, low-sugar medium, which facilitates rooting.

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Cotton (*Gossypium hirsutum* L.)

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Summary

Considering the economic importance of cotton in many developing and developed countries, there is an urgent need to accelerate the application of biotechnological tools to address the problems associated with the production of this crop and to improve the quality of fiber and seed. This requires a simple yet robust gene delivery/transformant recovery system. A protocol for the production of transgenic cotton plants was refined in our laboratory. It involves *Agrobacterium*-mediated transformation of cotton cells, selection of stable transgenic callus lines, and recovery of plants via somatic embryogenesis. A detailed description of the protocol is provided in this chapter.

Key Words: *Agrobacterium*; regeneration; somatic embryogenesis; transformation; transgenic cotton.

1. Introduction

Cotton, the most important source of natural fiber worldwide, is grown in more than 80 countries. In addition to the fiber, cottonseed is an important product of the plant that is used as cattle feed, either as seed or as meal following extraction of edible oil. Transgenic cotton developed to confer resistance against a class of insects through the expression of the Bt gene represents the first successful application of genetic engineering for commercial purposes (1,2). Bt cotton has lowered production costs by reducing the use of chemical pesticides, fuel, and labor input (3). In addition, this biotechnology product has had a positive impact on the environment and on human health by reducing pesticide use. Genetic engineering will continue to play an important role in improving both crop productivity and the quality of fiber and cottonseed.

The first two studies on cotton transformation were published in 1987 (4,5), 4 yr following the successful transformation of the model species tobacco. Despite early successes, there was a dearth of reports on transgenic cotton over

the next 15 yr. This was not owing to a lack of interest but because production of transgenic cotton remained extremely difficult, was highly inefficient, and required a high degree of tissue culture skills. The production of transgenic plants in any species involves two distinct and equally important steps. The first step entails transfer and stable integration of the transgene into the plant genome. The second step involves the recovery of a transgenic plant from the stably transformed cell. Given the difficulties involved in the production of transgenic cotton, a comprehensive study was undertaken in our laboratory to investigate both these aspects of transgenic cotton production (6). Although reports existed on gene gun-mediated (7,8) as well as *Agrobacterium*-mediated (4,5) transformation of cotton, our study (6) focused on the latter method since it does not require specialized equipment, is relatively inexpensive, and is more likely to result in single-copy transgenic events.

The choice of explant is critical in successfully obtaining transgenic plants since the cells within these tissues must be susceptible to *Agrobacterium* infection and must also be able to regenerate into healthy, fertile plants. Most published studies had used either hypocotyl segments (1,5,9,10) or cotyledon pieces (4,9) derived from a young seedling as tissue explants for transformation. However, the use of these explants necessitates passage of transformed cells through either a callus phase or a combination of callus and suspension cultures prior to induction of somatic embryogenesis for recovering transgenic plants. In addition to these tissue culture-based approaches, there are a few reports on the transformation of cells within shoot apices in cotton (11,12). Direct transformation of the “germline-progenitor” cells within the shoot apex has the obvious advantages of avoiding long, laborious tissue culture passage and decreasing both the length of time for the recovery of transformants and the somaclonal variations. Another major advantage of this system is that regeneration from shoot apices is genotype independent.

Various systems for obtaining transgenic cotton were thoroughly evaluated in our laboratory (6) using a reporter gene encoding green fluorescent protein (GFP) (13). GFP expression provided an excellent measure of the efficiency of T-DNA transfer into cotton cells and was also useful in understanding the timing and localization of transient transgene expression (6). Cells at the cut edge of the cotyledon segments proved to be the most susceptible to *Agrobacterium*-mediated transformation as indicated by transient GFP activity. This was followed by conversion of some of these transiently transformed cells to stable transformation events. Fewer cells at the cut surface of the hypocotyl showed transient GFP fluorescence compared with the cotyledonary tissue. The cells that showed transient activity were seen as a ring of fluorescent cells in the middle of the cut surface that appeared to be part of the vascular tissue; however, their true identity was masked by the hypersensitive response dis-

played by cells around them. Despite the lower rate of transient transformation, hypocotyl segments gave rise to several stable transgenic events that were seen as small fluorescent clusters growing at the cut surface of hypocotyls during selection on kanamycin-supplemented medium over 3 to 4 wk. Although hypocotyl segments showed a low level of transient activity compared with the cotyledons, these explants were still capable of producing several stable transgenic events (see **Note 1**). The efficiencies of cotyledonary petiole segments to yield stable transformation events were similar to those of the hypocotyl segments (see **Note 1**). Thus, the use of GFP as the reporter gene in combination with the neomycin phosphotransferase II (*nptII*) gene as a selectable marker showed that cotyledons, hypocotyls, and cotyledonary petioles are highly competent explants for *Agrobacterium*-mediated transformation. A single experiment involving 50 donor seedlings can yield several hundred independent transgenic events in the form of kanamycin-resistant calli.

The competence of shoot apices for *Agrobacterium*-mediated transformation was evaluated by cocultivating these with *Agrobacterium* under conditions that were found to be optimal for infection of the other three tissue explants. However, neither transient nor stable GFP expression in the “germline progenitor” cells within these tissues was observed in any of the experiments, each involving several hundred shoot apices. The results indicated that the efficiency of *Agrobacterium*-mediated transformation of apical meristems is extremely low in cotton.

In cotton, the mode of regeneration from callus or suspension cultures is via somatic embryogenesis (14–19). However, embryogenesis in the cultured tissue occurs at very low frequency and is highly genotype dependent. In addition, very few genotypes have been identified that are competent for regeneration (9,19–23). Even with genotypes that exhibit the best response, regeneration requires eight to ten subcultures on various media and can take as long as 6 to 10 mo to obtain plants following transformation. Problems are encountered at every step during regeneration. These include: (1) survival of transgenic events following excision from the hypocotyl, petiole, or cotyledon segments; (2) low efficiencies of the excised events forming friable calli, somatic embryogenesis, and germination of the somatic embryo into a normal plantlet with a proper shoot and root; and (3) extreme fragility of the plantlets during the transition from culture to soil. Thus, the production of stably transformed calli is an efficient process in cotton, as mentioned earlier; however, the recovery of healthy transgenic cotton plants was found to be highly inefficient.

We conducted a thorough examination of the factors impacting both transformation and regeneration and developed an efficient protocol for the production of transgenic cotton plants (6). The percentage of transgenic events obtained from hypocotyl and cotyledonary petiole explants (number of kana-

mycin-resistant, transgenic events obtained/number of explants cocultivated with *Agrobacterium* strain $\times 100$) ranged from 97 to 321% in various experiments. Although regeneration in cotton is possible through suspension cultures, we prefer to recover plants from callus cultures because this culture system requires less labor and equipment and there is less contamination. By using the protocol described in this chapter, regeneration efficiencies (number of transgenic lines regenerating into healthy plants/number of kanamycin-resistant culture lines $\times 100$) of up to 10% have been obtained. The method refined over the last 5 yr for producing transgenic cotton (cv. Coker 312) is presented in detail in this chapter.

For a thorough analysis of a transgenic trait, it is important to study the inheritance and stability of the phenotype over several generations. This necessitates identifying null segregants and the transgene-containing progeny in the segregating T_1 population and further identification of T_2 progeny that are derived from a homozygous T_1 parent. In several plant species such as *Arabidopsis* and tobacco, germination of seeds from transgenic lines on an antibiotic- or herbicide-supplemented medium (depending on the selectable marker gene used) provides an unambiguous identification of the null segregant or transgene-expressing progeny. However, in the case of cotton, several attempts to distinguish *nptII* gene-expressing progeny by germinating T_1 seeds on kanamycin-supplemented medium failed to provide consistent, clear-cut results. Therefore, an alternative method was devised that was based on callus induction in cotyledon segments in the presence of kanamycin. Its reliability was confirmed by polymerase chain reaction (PCR) analysis on the seedlings that were used for the callus induction assay. This method was used successfully in our laboratory (24) and is described in detail in this chapter. It provides an unambiguous proof of *nptII* gene expression in cotton seedlings and may be applicable to other species.

2. Materials

With the exception of a few chemicals (as stated), the materials can be purchased from Sigma. All media are autoclaved at 121°C for 20 min after adjusting the pH and after the addition of the gelling agent. Autoclaved media should be cooled to <60°C before adding filter-sterilized antibiotics or acetosyringone.

2.1. *Agrobacterium* Media

1. YEP: 10 g/L Bacto peptone (Difco), 5 g/L NaCl, 10 g/L Bacto Yeast Extract (Difco), pH 7.0, 1.5% Bacto agar (Difco). Aliquot in small batches in bottles, autoclave, and store at room temperature. When required, melt in microwave, add necessary antibiotics, and pour into plates.

2. YEP liquid: 10 g/L Bacto peptone, 5 g/L NaCl, 10 g/L Bacto yeast extract, pH 7.0. Autoclave and store at room temperature.
3. PIM (preinduction medium): 10 g/L glucose, 14.62 g/L morpholinoethanesulphonic acid (MES), 20 mL/L sodium phosphate buffer (0.1 M, pH 5.6), 50 mL/L AB salts stock (20X), pH 5.6. Filter-sterilize, aliquot in 10-mL portions, and store at 4°C.
4. AB salts stock (20X): 20 g/L NH_4Cl , 6 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 3 g/L KCl, 0.264 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.05 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$. Autoclave and store at 4°C.
5. Sodium phosphate buffer (0.1 M, pH 5.6): 2.759 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ /200 mL water (solution A); 2.839 g Na_2HPO_4 /200 mL water (solution B). Add solution B to solution A in a stepwise manner until the pH is brought to 5.6, autoclave, and store at room temperature.

2.2. Cotton Tissue Culture Media and Plant Growth Media

1. Cotton seeds (*Gossypium hirsutum* L., cv. Coker 312).
2. MSO: 4.31 g/L Murashige and Skoog (MS) salts (25) (MS basal salts mixture; PhytoTechnology Laboratories, cat. no. M524), 2% glucose, pH 5.8, 0.2% Phytagel.
3. P1-AS: 4.31 g/L MS salts, 100 mg/L myoinositol, 0.4 mg/L thiamine HCl, 5 mg/L N^6 -(2-isopentenyl)adenine (2ip; PhytoTechnology Laboratories, cat. no. D525 or Sigma, cat. no. D-7674), 0.1 mg/L α -naphthaleneacetic acid (NAA), 3% glucose, 1 g/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, pH 5.8, 0.2% Phytagel, 50 μM acetosyringone (AS; Aldrich, cat. no. D13440-6; AS stock is made at 10 mg/mL in 70% ethanol and stored at -20°C; add 1 mL of AS stock to 1 L of P1).
4. P1-c4k50: 4.31 g/L MS salts, 100 mg/L myoinositol, 0.4 mg/L thiamine HCl, 5 mg/L 2ip, 0.1 mg/L NAA, 3% glucose, 1 g/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, pH 5.8, 0.2% phytagel, 400 mg/L carbenicillin (PhytoTechnology Laboratories, cat. no. C346), 50 mg/L kanamycin (Invitrogen, cat. no. 11815-032).
5. P7-c4k50: 4.31 g/L MS salts, 100 mg/L myoinositol, 0.4 mg/L thiamine HCl, 0.1 mg/L 2ip, 5 mg/L NAA, 3% glucose, 1 g/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, pH 5.8, 0.2% Phytagel, 400 mg/L carbenicillin, 50 mg/L kanamycin.
6. MSBOK-c2: 4.31 g/L MS salts, 100 mg/L myoinositol, 1 mg/L nicotinic acid, 10 mg/L thiamine HCl, 1 mg/L pyridoxine HCl, 1.9 g/L KNO_3 , 3% glucose, 1 g/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, pH 5.8, 0.2% Phytagel, 200 mg/L carbenicillin.
7. EG3: 2.16 g/L MS salts, 0.5% glucose, 100 mg/L myoinositol, 0.4 mg/L thiamine HCl, 0.01 mg/L NAA, pH 5.9, 0.2% Phytagel.
8. MS3: 2.16 g/L MS salts, 0.5% glucose, 0.14 mg/L thiamine HCl, 0.1 mg/L pyridoxine HCl, 0.1 mg/L nicotinic acid, pH 5.8, 0.08% Phytagel, 0.6% Bacto agar (Difco).
9. P1-k200: 4.31 g/L MS salts, 100 mg/L myoinositol, 0.4 mg/L thiamine HCl, 5 mg/L 2ip, 0.1 mg/L NAA, 3% glucose, 1 g/L $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, pH 5.8, 0.2% Phytagel, 200 mg/L kanamycin.
10. Sunshine LP5 soil mix: 70 to 80% Canadian sphagnum peat moss + fine perlite + dolomite limestone + gypsum + wetting agent (Sun Grow Horticulture Canada Ltd.).

11. MetroMix 700 soil mix: 50 to 60% bark + Canadian sphagnum peat moss + horticulture grade vermiculite + dolomite lime stone + wetting agent (Sun Grow Horticulture Canada Ltd.).

3. Methods

3.1. Preparation of *Agrobacterium Inoculant*

1. Streak the *Agrobacterium tumefaciens* strain harboring a binary vector with the gene of interest on antibiotic-supplemented YEP plates and incubate for 2 to 3 d at 28°C (see **Note 2**).
2. Inoculate five colonies into five different test tubes each containing 2 mL of YEP liquid medium (supplemented with appropriate antibiotics). Grow cells at 28°C for about 30 h on a shaker (200 rpm).
3. Pool bacterial cultures in a 15-mL centrifuge tube. Centrifuge at 2060g for 15 min. Remove as much supernatant as possible with a sterile pipet and resuspend the bacterial pellet in 10 mL of PIM with 100 μ M acetosyringone (add 20 μ L of 10 mg/mL AS stock to 10 mL of PIM). Be sure to break up bacterial clumps by vortexing. Transfer the suspension to a 125-mL flask. Grow cells at 28°C for approx 24 h on a shaker (200 rpm).
4. Use 1 mL of suspension to check OD at 600 nm. It should be in the range of 1.6 to 1.9. Add 18 μ L of acetosyringone stock to the 9 mL of the remaining suspension before using the *Agrobacterium* culture for cocultivation (see **Note 3**).

3.2. Aseptic Seed Germination

1. Rinse 50 delinted seeds under running tap water for 3 h.
2. Treat the seeds with 70% ethanol for 1 min and wash twice with sterile distilled or deionized water (DW).
3. Sterilize the seeds by shaking them in a flask containing 100 mL of 20% commercial bleach (+ 2 drops of Tween-20) for 5 min under vacuum followed by 15 min without vacuum. Rinse three times with sterile DW (see **Note 4**).
4. Germinate one seed per jar or Magenta box on MSO (25 mL/jar; 50 mL/Magenta box) at 28°C, under light (70 μ mol/m²/s, 16-h photoperiod) for 10 d.

3.3. Transformation

1. Cut 3- to 4-mm-long segments from either hypocotyl or cotyledonary petiole. Place 10 to 12 segments horizontally on sterile filter paper (7 cm diameter, Fisher Scientific, cat. no. 09-801A) over P1-AS medium.
2. Apply 5 μ L of acetosyringone-induced *Agrobacterium* suspension to each cut surface. Keep the plates under light (70 μ mol/m²/s, 16-h photoperiod) at 25°C for 3 d for cocultivation.
3. Transfer hypocotyl/petiole pieces to P1-c4k50. Keep the plates under light (70 μ mol/m²/s, 16-h photoperiod) at 28°C for 3 to 4 wk without subculture (see **Note 5**).

3.4. Selection/Proliferation

1. Carefully excise individual calli, representing individual transgenic events, growing at the cut surface of the explant (see **Notes 6** and **7**).
2. If the calli are 3 mm or larger, place these on fresh P7-c4k50 plates and grow for 4 wk at 28°C but under a reduced light intensity of approx 10 $\mu\text{mol}/\text{m}^2/\text{s}$ (16-h photoperiod).
3. If the excised calli are smaller than 3 mm, culture these for 7 d on P1-c4k50 plates at 28°C under a light intensity of approx 10 $\mu\text{mol}/\text{m}^2/\text{s}$ (16-h photoperiod) prior to transferring them to P7-c4k50 plates (see **Note 8**).
4. Subculture again on the P7-c4k50 medium, and grow the callus cultures under the same conditions for 4 wk.
5. Discard the lines that produce only hard, compact, and green-colored callus. Select lines that are producing friable and pale cream-colored callus at the periphery. Divide only if necessary at this stage. Transfer to fresh P7-c4k50 and culture under the same conditions for an additional 4-wk period.

3.5. Regeneration (Somatic Embryogenesis), Embryo Germination, and Plantlet Development

1. Again select lines that are producing friable and pale cream-colored calli. Separate friable (potentially embryogenic) callus, transfer to MSBOK-c2 and culture at 28°C under a light intensity of approx 10 $\mu\text{mol}/\text{m}^2/\text{s}$ (16-h photoperiod) for the next 4 to 16 wk. Maintain cultures with monthly transfer to fresh medium until somatic embryos appear (see **Note 9**).
2. Select embryos that are at least 7 to 8 mm long and place on filter paper (same type as used during cocultivation) over EG3 medium in deep Petri dishes (25 × 100 mm). Do not transfer more than eight embryos per dish.
3. Maintain embryos for 3 to 6 wk under light (70 $\mu\text{mol}/\text{m}^2/\text{s}$, 16-h photoperiod) at 25°C.
4. After 6 wk, evaluate embryos for the presence of shoot apices. Discard the embryos that have failed to develop shoot apices.
5. Transfer plantlets (having well-defined shoot apices and roots) that are smaller than ½ inch to fresh EG3 medium without the filter paper and maintain under light (70 $\mu\text{mol}/\text{m}^2/\text{s}$, 16-h photoperiod) at 25°C.
6. Select plantlets with approx ½- to 1-inch-long shoots, a few visible true leaves, and some roots. Transfer these to jars containing MS3 medium and maintain under light (70 $\mu\text{mol}/\text{m}^2/\text{s}$, 16-h photoperiod) at 28°C for further growth and root establishment (see **Note 10**).

3.6. Transfer to Soil

1. Transfer plants to Sunshine LP5 soil mix in 500-mL size pots when the shoots are 2.5 to 3 inches in length and show significant root development. Keep plants under low-level lighting (20–30 $\mu\text{mol}/\text{m}^2/\text{s}$) and high humidity under a clear plastic dome (see **Note 11**). After 2 wk, remove the dome and maintain under the same lighting conditions for an additional week.

2. Transfer healthy plants that have grown to a height of 5 to 6 inches to MetroMix 700 soil mix in 20-L size pots and grow the plants in a greenhouse to maturity ($900 \mu\text{mol}/\text{m}^2/\text{s}$, 14-h photoperiod) (see **Notes 12–15**).
3. Isolate DNA from a young leaf for molecular analysis to confirm the transgenic status of the plant.

3.7. Kanamycin Resistance Test

1. Aseptically germinate cottonseeds obtained from T_0 transgenic plants as described earlier.
2. Excise ten segments ($0.5 \times 0.5 \text{ cm}$) from a single cotyledon of a 10-d-old seedling and place on P1-k200 medium. The remaining seedling can be transferred to soil in a small pot until its transgenic status is ascertained.
3. Keep the plates at 28°C under a 16-h photoperiod ($70 \mu\text{mol}/\text{m}^2/\text{s}$).
4. Score the cotyledonary segments, 21 d post culturing, for the presence of kanamycin-resistant callus at the edges by visualizing the tissues under a stereo microscope.
5. If none of the cotyledon segments obtained from a seedling produce kanamycin-resistant callus, it is considered a null segregant. If cotyledon segments from a seedling produce callus on selection plates, the seedling is considered transgenic (see **Note 16**).
6. Similarly, test T_2 seeds from a segregating T_1 population to identify the progeny obtained from a T_1 homozygous parent (see **Note 17**).

4. Notes

1. Stable transformation events in the form of kanamycin-resistant calli arising at the cut surface of three different explants are shown in **Fig. 1**. In some cases we have observed as many as ten independent transgenic events from a single cut surface of hypocotyl or petiole segments.
2. *A. tumefaciens* strain LBA4404 provides higher transformation efficiencies in cotton as compared with the strain EHA105 (**6**).
3. Transformation efficiencies are improved significantly by using the *Agrobacterium* inoculant preparation protocol described here. Alternatively, it is possible simply to grow *Agrobacterium* on YEP plates, harvest the cells with a loop, and resuspend them in PIM+AS before cocultivation. This simpler procedure eliminates the need for a shaker.
4. It is possible to use fuzzy seeds (ginned seeds that have not been acid-delinted) by modifying the sterilization protocol. This involves soaking the seeds overnight in running tap water followed by sterilization with 40% commercial bleach (+ 2 drops of Tween-20) under vacuum for 40 min. After rinsing, the embryo can be isolated for germination by carefully cutting and removing the seed coat.
5. Although the transformation/regeneration protocol described is for hypocotyl and cotyledonary petiole segments, it can also be used for cotyledon segments. Individual transgenic events arising from the edges of the cotyledon segments can be used to obtain plants by following the method described in this chapter.

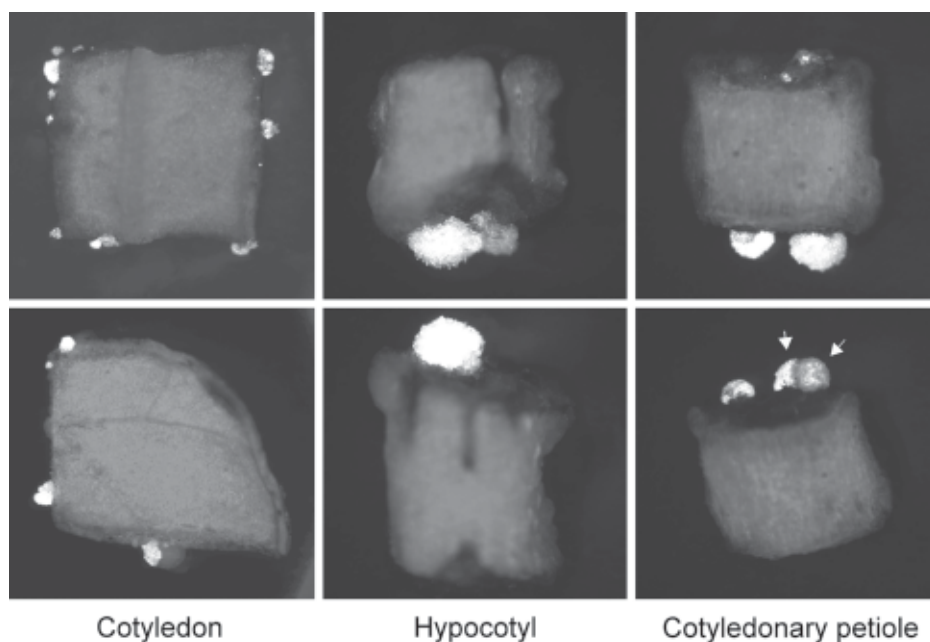


Fig. 1. Two examples each from cotyledon, hypocotyl, and cotyledonary petiole explants showing GFP-expressing, kanamycin-resistant calli. The arrowheads in the lower right image indicate two separate transgenic events growing side by side and merging with each other. The images were captured 3 wk following inoculation with an *Agrobacterium* strain harboring pBINmGFP5-ER (6,13). In order to depict the outline of the explants in a black and white image, the emission filter that also permitted visualization of the red-colored autofluorescence from chlorophyll was used.

6. Often, more than one transgenic event is observed growing at the cut surface of the hypocotyl or the petiole segments. The longer these are permitted to grow on the explant, the greater the likelihood that two or more transgenic events will converge with each other. In this situation, the possibility remains that the two plants obtained from the same excised callus tissue may have arisen from separate transgenic events.
7. Recovery of transgenic events from hypocotyl and cotyledonary petiole segments and efficiencies of regenerating plants from these lines are presented in **Table 1**. The results presented in the table illustrate clearly that the efficiencies of obtaining stable transformation events from hypocotyl and cotyledonary petiole explants are very high in cotton; however, the regeneration efficiencies are lower in comparison. As is the case with the transformation of any species, experiment-to-experiment variability is also observed with cotton. This necessitates performing several small-scale experiments for a given construct rather than a few large-scale ones.

Table 1
Kanamycin-Resistant Callus Lines from Hypocotyl
and Cotyledonary Petiole Explants and Regeneration of Plants

Explant	Experiment no.	No. of explants infected	No. of Kan ^R calli excised	No. of lines regenerated into plants
Hypocotyl	1	110	161	1
	2	380	620	2
	3	40	40	7
	4	100	180	0
	5	60	83	0
	6	40	70	8
	7	40	120	8
	8	150	200	4
	9	190	328	12
	10	160	156	3
	11	NC	308	33
	12	160	322	10
	13	180	397	15
	14	360	1156	87
Cotyledonary petiole	1	150	170	3
	2	180	450	0
	3	80	145	5
	4	200	564	8
	5	150	236	16
	6	330	376	3
	7	100	199	9
	8	100	232	22

NC, not counted.

8. The additional week of culture on P1-c4k50 medium improves the survival of small-sized transgenic events; however, this may delay embryogenesis.
9. The growth of and differentiation in individual transgenic events is highly asynchronous, necessitating regular monitoring of the cultures. Transfer somatic embryos developing at any point during the regeneration phase to EG3 medium for embryo germination.
10. It was possible to obtain regeneration efficiencies higher than those shown in [Table 1](#) by continuing to subculture all the transgenic lines obtained in an experiment until they turned embryogenic. However, this practice is not recommended

since it results in a higher rate of somaclonal variations owing to the increased length of time in culture.

11. Mild growth conditions to acclimatize the transgenic plants following the transfer to soil can be attained by maintaining the plants on the laboratory bench under room lighting under a clear plastic dome to maintain high humidity.
12. In addition to the commercial soil mixtures described here, cotton plants may be grown in other suitable soil mixtures. In the greenhouse, cotton plants are fertilized once a week with 20-20-20 NPK mix (Peters® Professional Fertilizer, Scotts Co.) until flowering and then with 8-45-14 NPK mix (Peters® Professional Fertilizer). The plants are also fertilized every 2 wk with a micronutrient mix (Peters® Professional Soluble Trace Elements Mix, Scotts Co.). Both wild-type and transgenic plants are equally prone to various insect pests in the greenhouse. Appropriate insecticide treatment can be used to control the insect pests. We use Marathon II™ (active ingredient [AI]: 21.4 % imidacloprid, Olympic Horticultural Products Co.) to control whiteflies, Talstar-GH™ (AI: 7.9% bifenthrin, Whitmire Micro-Gen) to control aphids, Avid™ (AI: 1.8% abamectin, Syngenta Crop Protection, Inc.) + Talstar-GH™ to control spider mites, and Conserve™ (AI: 11.6% spinosad, Dow AgroSciences LLC) to control thrips.
13. Less than 5% of the transgenic plants obtained using the protocol described here exhibit abnormal morphology owing to somaclonal variation. Under greenhouse conditions we have been able to obtain seeds in 4 to 5 mo, and 60 to 75% of the transgenic plants obtained in various experiments have been fertile.
14. It may be possible to use the protocol described here for cultivars other than Coker 312.
15. The *nptII* gene in combination with kanamycin provided a highly effective selection system to produce transgenic cotton plants. Molecular analyses (Southern and PCR) suggested that no untransformed plants (escapes) were recovered using the selection scheme described in this chapter. The *hpt*/hygromycin B and *bar*/phosphinothricin selection systems were found to be less effective in cotton.
16. The cotyledon callusing assay will not provide accurate results if the transgene has undergone partial or complete silencing. In that case, molecular proof will be required to identify transgene-containing progeny.
17. Since only one cotyledon is used for this assay, upon confirmation of the transgenic status or the homozygosity status, the remaining seedling can be grown to maturity to obtain seeds.

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Indian Mustard [*Brassica juncea* (L.) Czern.]

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Summary

All economically important *Brassica* species have been successfully transformed using *Agrobacterium tumefaciens*. Although different tissues have been used as explants, hypocotyls remain the most desirable explants for *Brassica* tissue culture owing to their amenability to regeneration. Young explants excised from 3- to 4-d-old seedlings have exhibited optimal regeneration potential; the addition of adjuvants such as silver nitrate to the selection medium is necessary to achieve high efficiency of transformation. This chapter describes an *Agrobacterium*-mediated transformation protocol for Indian mustard based on inoculation of hypocotyls. The selectable marker gene used encodes for neomycin phosphotransferase II (*nptII*), and the selection agent is kanamycin.

Key Words: *Agrobacterium tumefaciens*; Indian mustard; genetic transformation.

1. Introduction

Transformation systems have been developed for almost all economically important species of *Brassica* (1–6). A variety of transformation methods along with factors influencing transformation efficiency as well as progress in understanding the cellular and molecular biology of *Brassica* species have recently been reviewed (7–9). *Agrobacterium tumefaciens*-mediated transformation is considered the most widely used and efficient method for most species in the *Brassica* genus.

Regeneration has been induced using various tissue explants such as cotyledons (10–12), hypocotyls (13), peduncle segments (14), leaves (15), thin cell layers of epidermal and subepidermal cells (16), roots (17), and protoplasts (18–21). For most *Brassica* species, hypocotyl segments remain the most desirable target explants for transformation as they are highly amenable to regeneration. As regeneration is dependent on the age of the explant (22), it has been reported that young explants excised from 3- to 4-d-old seedlings yield

optimal frequencies of regeneration (10). Furthermore, the presence of the ethylene inhibitor silver nitrate is deemed essential for promoting high frequencies of regeneration in *Brassica* (23–28), particularly when kanamycin is used as a selectable marker (29). Vitrification (hyperhydricity) of regenerated shoots is a problem occasionally observed in tissue culture that can be prevented by simultaneously lowering both the cytokinin and silver nitrate levels and increasing the agar content (4). In this chapter, we describe an *Agrobacterium*-mediated transformation protocol for *Brassica juncea* (L.) Czern., Indian mustard, based on previous reports and our current experience in this area. In our work, we have used hypocotyls, excised from young seedlings and cut into three to four pieces, as explants for transformation. The selectable marker gene *nptII*, coding for neomycin phosphotransferase II and kanamycin resistance, is used. The transformation efficiency (percent of explants that gave rise to rooted transgenic plants on kanamycin-containing rooting medium) was 16%. Following Southern blot analysis, 87% of putative transformants were positive for the presence of both *nptII* and the target transgene.

2. Materials

2.1. *Agrobacterium tumefaciens* Strain and Medium

1. *A. tumefaciens* strain GV3101, harboring the binary plasmid pBI121 (Clontech), is used in this chapter. This plasmid contains *nptII* as a selectable marker gene (see Note 1).
2. 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, and then cool down to 55°C before adding 100 mg/L kanamycin (see Note 2).

2.2. Plant Material

Indian mustard (*Brassica juncea*, accession no. 426308) seeds (200–300 per transformation).

2.3. Media Stock Solutions

All stocks are made with distilled-deionized water (ddH₂O) and stored at 4°C unless otherwise noted. All antibiotic solutions were filter-sterilized and added to media after autoclaving.

1. 5% Hypochlorite solution.
2. Murashige and Skoog (MS) medium (PhytoTechnology Laboratories, Shawnee Mission, KS).
3. 6-Benzylaminopurine (BA; Sigma, St. Louis, MO), 2 mg/mL stock. 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 ddH₂O. Store at 4°C for up to 6 mo.

4. Naphthalene acetic acid (NAA; Sigma), 1 mg/mL stock. 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 ddH₂O. Store at 4°C for up to 6 mo.
5. Indole-3-butyric acid (IBA; PhytoTechnology Laboratories), 1 mg/mL stock. Dissolve 10 mg IBA in 2 mL 1 N NaOH in a beaker with a magnetic stir bar. Bring final volume to 10 mL with ddH₂O. Store below 0°C for up to 6 mo.
6. Acetosyringone (3,5-dimethoxy-4-hydroxy-acetophenone; Sigma), 200 μ M stock. Dissolve 392 mg of acetosyringone in 10 mL dimethylsulfoxide (DMSO; Sigma). Store at 4°C. Add to media after autoclaving.
7. Silver nitrate (AgNO₃; Sigma), 30 μ M stock. Dissolve 51 mg of AgNO₃ in 10 mL ddH₂O, and store at 4°C. Add 1 mL/L to medium before autoclaving.
8. Kanamycin monosulfate (PhytoTechnology Laboratories), 50 mg/mL stock. Dissolve 500 mg kanamycin monosulfate in 8 mL ddH₂O in a beaker with a magnetic stir bar. Bring final volume to 10 mL with ddH₂O. Filter-sterilize, and divide into 1-mL aliquots. Store at -20°C for up to 6 mo.
9. Vancomycin (Sigma), 50 mg/mL stock. Dissolve 500 mg vancomycin in 8 mL ddH₂O in a beaker with a magnetic stir bar. Bring final volume to 10 mL with ddH₂O. Filter-sterilize, and divide into 1-mL aliquots. Store at -20°C for up to 6 mo.
10. Cefotaxime sodium salt (Sigma), 200 mg/mL stock. Dissolve 2 g cefotaxime sodium salt in 8 mL ddH₂O in a beaker with a magnetic stir bar. Bring final volume to 10 mL with ddH₂O. Filter-sterilize, and divide into 1-mL aliquots. Store at -20°C for up to 6 mo.
11. Soil mix: Sunshine Mix #1 (Sun Gro Horticulture Inc.).

2.4. Tissue Culture Media

1. Germination medium: half-strength MS salts and vitamins (PhytoTechnology Laboratories) supplemented with 20 g/L sucrose, 8 g/L agar (PhytoTechnology Laboratories), pH adjusted to 5.8 using 1 N NaOH, autoclaved for 20 min at 121°C, and poured into deep (100 $\times$ 150-mm) Petri plates.
2. Cocultivation medium: full-strength MS salts and vitamins supplemented with 4 g/L agar, 10 g/L sucrose, 10 g/L glucose, 10 g/L mannitol, 2 mg/L BA, 0.1 mg/L NAA, and 200 μ M acetosyringone (see **Note 3**), pH 5.8
3. Selection medium A: full-strength MS salts and vitamins supplemented with 10 g/L agar (see **Note 4**), 10 g/L sucrose, 10 g/L glucose, 10 g/L mannitol, 250 mg/L cefotaxime, 100 mg/L vancomycin, 50 mg/L kanamycin (see **Note 5**), 2 mg/L BA, 0.1 mg/L NAA, and 30 μ M AgNO₃ (see **Note 6**), pH 5.8.
4. Selection medium B: selection medium A plus 10% coconut water (Sigma).
5. Rooting medium: full-strength MS salts and vitamins supplemented with 4 g/L agar, 30 g/L sucrose, 100 mg/L cefotaxime, 50 mg/L kanamycin, and 1 mg/L IBA, pH 5.8.
6. Liquid MS medium: half-strength MS dissolved in ddH₂O, and autoclaved for 20 min at 121°C.

3. Methods

3.1. Seed Sterilization and Germination

1. Sterilize Indian mustard seeds (200–300 per transformation) in 5% hypochlorite solution for 10 min, and then rinse four times for 10 min in sterilized ddH₂O, all on a rocking platform.
2. Sow 50 seeds per Petri plate (100 × 150 mm) on germination medium, and grow for 3 d ([Fig. 1A](#)). All tissue cultures are grown at 25°C under continuous light (25 $\mu\text{mol}/\text{m}^2/\text{s}$).

3.2. *Agrobacterium tumefaciens* Preparation

1. Streak *Agrobacterium* cells from a permanent glycerol stock onto solid LB with 100 g/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 recultured in 50 mL liquid LB medium until an OD₆₀₀ reading of 1.3 to 1.5 is obtained.
4. Before preparing and inoculating Indian mustard explants, the bacterial culture is centrifuged at 6000g 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 OD₆₀₀ = 0.6 with liquid MS.

3.3. Explant Preparation and Inoculation

1. All manipulations involving plant and bacterial materials were done in a laminar flow hood under sterile conditions.
2. Prepare explants by removing root and leaf tissues with a sterile razor blade, and place hypocotyls into a sterile Petri plate. Cut hypocotyls into smaller segments (5–10 mm in length), and immerse them into liquid MS until all explants are prepared ([Fig. 1B](#)) (see [Note 7](#)).
3. When explants are prepared, replace liquid MS with the bacterial suspension (OD₆₀₀ = 0.6), and inoculate for 1 h ([Fig. 1C](#)).
4. After immersion in the bacterial suspension, blot hypocotyls dry with sterile filter paper to remove excess liquid containing *Agrobacterium*, and place them (approx 50 explants per Petri plate) on cocultivation medium at 25°C under continuous light (25 $\mu\text{mol}/\text{m}^2/\text{s}$).

3.4. Selection and Plant Regeneration

1. After 2 d on cocultivation medium, wash hypocotyls by dipping them in a liquid MS medium for 45 min at room temperature in a laminar flow hood, blot dry, and then transfer to selection medium A ([Fig. 1D](#)).
2. After 11 d, transfer hypocotyls to selection medium B. Within days, a callus tissue will develop along both ends of the explant, and pinkish buds are likely to be observed (see [Note 8](#); [Fig. 1E](#) and [F](#)).

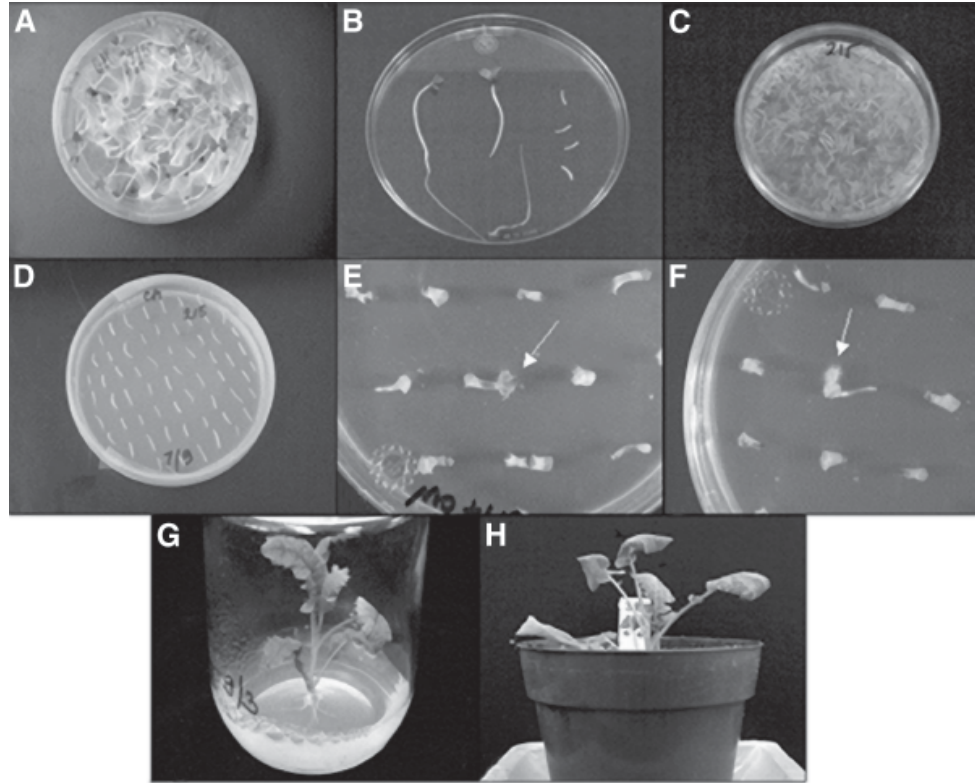


Fig. 1. *Agrobacterium*-mediated transformation of hypocotyls of *Brassica juncea* L. (A) Seed germination in deep Petri dishes. (B) Explant preparation. (C) *Agrobacterium* inoculation. (D) Placing hypocotyls on selection medium. (E) Shoot regeneration via callus and pink-colored shoot bud stage. (F) Root regeneration via callus stage. (G) Rooting of regenerated shoots. (H) Transfer of well-developed plantlets to soil.

3. When either calli or shoot buds appear, they must be separated from the explant, transferred to either a new Petri plate or a glass jar containing medium B, and placed in direct contact with the medium (see **Note 9**).
4. Transfer established shoots to rooting medium. After approx 4 wk, plantlets (4–5 cm with well-established roots) are transferred to soil and acclimatized in the greenhouse (**Figs. 1G and H**) (see **Note 10**). Almost 100% of shoots develop roots.
5. Leaf tissue will be collected from young plantlets and subjected to molecular analyses, including polymerase chain reaction (PCR) and Southern blotting to confirm the presence and integration of the transferred gene.

3.5. Greenhouse Acclimatization and Care

1. Gently remove plants with well-formed root systems from the culture vessel and wash the medium from the roots using lukewarm tap water (see **Note 10**).
2. Transfer each plantlet to a 15-cm plastic pot containing a thoroughly wetted soil and cover with a plastic container or plastic bag secured to the pot (see **Note 11**).
3. Transfer plants to shaded area of a greenhouse (see **Note 12**).
4. After 3 to 4 d, cut a few small holes in the plastic bag or raise the plastic container a small amount. Repeat this each day for 1 wk for a gradual acclimation. Remove the cover on the 8th d.

The survival rate of transgenic plants following acclimatization is 95 to 100%. Within 3 to 4 mo, plants will begin to flower, and seed can later be harvested. There were no fertility problems, and seed set rates in transgenic plants are not different from those of control plants.

4. Notes

1. The selectable marker gene used was *nptII* along with kanamycin at 50 mg/L as the selection agent for screening putative transformants and to allow for their rooting. It is necessary to determine the critical level of the selectable agent in the selection and rooting media that will only allow for survival of transformed plantlets.
2. The selective agent depends on the selectable marker gene.
3. Application of acetosyringone has increased transformation frequencies in many species (7). It is used to induce virulence genes in *Agrobacterium* (30,31).
4. The amount of agar in the selection medium is increased to 10 g/L owing to observed problems of hyperhydricity (4).
5. All antibiotics are added to autoclaved media from previously prepared stocks.
6. Silver nitrate is an ethylene inhibitor necessary for *Brassica* regeneration (26).
7. Maintain hypocotyls in liquid MS until they are ready for inoculation to prevent them from drying. Cutting hypocotyls to smaller pieces (5 mm) is very important to allow for their placement in contact with the selection medium. Longer pieces have a tendency to curl up, and the margins where calli develop are no longer in contact with the selection medium.

8. Occasionally root development is observed along ends of explants. This is attributed to residual root cells of the radicle of the original explant.
9. It is important to transfer explants weekly (no more than 2 wk) to fresh medium to obtain high efficiency of regeneration of putative transformants.
10. Washing the medium from the roots reduces the chance of bacterial and fungal growth that may kill the plantlet once it is placed in soil.
11. Pots with plantlets should be covered with either a clear plastic bag or a clear plastic covering, put into a tray, and moved to the greenhouse.
12. If new transfers to soil are placed in direct sunlight, heat will build up under the cover and kill the plant.

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Sunflower (*Helianthus annuus* L.)

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Summary

Sunflower (*Helianthus annuus* L.) is considered one of the recalcitrant species in terms of transformation and regeneration. A routine transformation system of this crop requires competent cell cultures for efficient plant regeneration as well as an effective method for gene delivery. A transformation system was developed by an *Agrobacterium tumefaciens*-mediated method using split mature embryonic axis explants from the Ha89 genotype. Mean transformation efficiency obtained (measured as PCR+ plants/treated explants) varied from 1 to 5.2% depending on the use of the EHA105 or the C58 strain containing a plasmid with a gene of agronomic interest. The system developed has applicability to several *Agrobacterium* strains and plasmids with both reporter genes or genes of agronomic interest. Plants obtained with this protocol were confirmed by PCR and Southern blot. Stable inheritance of transgenes was successfully followed until generation T₄ in several independent lines.

Key Words: *Agrobacterium tumefaciens*; *Helianthus annuus* L.; transgenic plants; meristem transformation; embryonic axis; sunflower transformation.

1. Introduction

Sunflower is the world's third most important oil-producing crop, accounting for about 13% of the total world edible oil production; it has been the object of several biotechnological improvements in the last few years. Sunflower is one of the most difficult species to be genetically transformed since for most explants, cells that are competent for regeneration are not competent for transformation (1).

Several groups have reported different approaches to overcoming this problem (explant treatments, vectors including hormone-encoding genes, transformation delivery systems including *Agrobacterium*, gene gun, protoplast, or combination approaches). Meristems derived from split mature embryonic axis

were shown to be a suitable explant for an efficient, routine protocol via *A. tumefaciens* (2). Most of the reported work used *gus* and *nptII* as visual and selective marker genes, respectively. Schrammeijer et al. (2), started with this kind of explant (meristems from mature embryos) and reported 0.1% transformation efficiency (TE) using strains EHA101 and LBA4404. Bidney et al. (3) reported 0.05% TE combining the coculture with gold particle bombardment. Other authors treated the Ha300b embryo explant with macerating enzymes in order to increase the levels of plant elicitor (4) or other plant cell wall repair factors (5) for activation of *Agrobacterium vir* genes.

The use of meristems as explants in a transformation protocol implies that chimerical shoots and plants are obtained. The percentage of chimerical tissue depends on the extent of the area that will be covered by *Agrobacterium* infection. An extensive description of the transformed tissue distribution was made by Burrus et al. (6), comparing the results obtained with whole and divided meristems using the Ha300b line and the GV2260 strain. Different authors reported on the use of meristems or plantlet apical tissues. Knittel et al. (7) reported 3% TE combining bombardment with coculture (LBA4404 strain) in explants of LG60 and LG61 genotypes obtained from 2-d-old germinated seeds followed by 5 d of in vitro culture. Rao and Rohini (8) simplified the protocol using an explant consisting of 2-d-old seedlings deprived of one of the cotyledons and reported 2% TE with the LBA4404 *Agrobacterium* strain and KBSH1 sunflower genotype. Other efforts were made to increase the regeneration potential in the meristematic area, like bombardment with the *ipt* gene, which codes for an isopentenyl transferase involved in the biosynthesis of cytokinins in plants, to induce transient expression and consequently an increase in the number of shoots per explant; transient GUS expression in this case was 6% (9).

The protocol described in this chapter uses *A. tumefaciens* strains EHA105 or C58 and the split embryo axis as explants from Ha89 sunflower genotype (10). Using kanamycin selection on this protocol, we obtained transgenic plants with 1% (for EHA105 strain) to 5% (for C58 strain) TE, namely, 1 to 5 polymerase chain reaction (PCR)-positive plants from 100 explants infected by *Agrobacterium*.

2. Materials

2.1. Genotypes

Public line Ha89 seeds are used from field-grown plants (see Note 1).

2.2. *Agrobacterium* Strain and Plasmids

1. *A. tumefaciens* strains EHA105 or C58 (see Note 2).
2. Plasmid pBI121.2 (Clontech, Palo Alto, CA): contains the *gus* gene under the CaMV 35S promoter and the *nptII* gene under the *nos* promoter and terminator.

2.3. *A. tumefaciens* Culture Media

1. YEP medium: 5 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl, and 12 g/L Bacto agar, adjusting to pH 7.0 with 1 N NaOH. The medium is sterilized by autoclaving and stored at room temperature (RT). Adequate filter-sterilized antibiotics are added just before pouring into 90-mm Petri dishes.
2. Minimum A liquid medium (Min A): 10.5 g/L K_2HPO_4 , 4.5 g/L KH_2PO_4 , 1 g/L $(NH_4)_2SO_4$, 0.5 g/L sodium citrate $\cdot$ 2H $_2$ O, 0.2 g/L $MgSO_4\cdot 7H_2O$, 2 g/L glucose, pH 7. Sterilize by autoclaving and store at 4 to 8°C. Adequate filter-sterilized antibiotics are added just before starting the bacterial culture.
3. Kanamycin monosulfate: 50 mg/mL stock solution in water, filter-sterilized (0.2- μ m syringe filter) and stored in 1-mL aliquots at -20°C. Working concentration is 50 mg/L (for plant and bacteria growing media).
4. Rifampicin: 100 mg/mL stock solution in dimethyl sulfoxide (DMSO) stored in 1-mL aliquots at -20°C. Working concentration is 100 mg/L.
5. Acetosyringone (20 mM): dissolve 100 mg of acetosyringone in 12.5 mL 96% ethanol. Add 25.5 mL water to final volume of 40 mL. Working concentration is 100 μ M.

2.4. *Ha89* Sunflower Explant Culture Media

All media are sterilized by autoclaving in 1-L flasks at 121°C, 1.2 bar for 15 min and stored at RT. Growth regulators, antibiotics, and vitamins are added to the medium after autoclaving. Media are aliquoted as 10 mL/tube under the flow hood in autoclaved culture tubes (10 cm height and 2.5 cm width, covered with cotton cups). Store at 4 to 8°C.

1. Medium MSi: 1X Murashige and Skoog macro, micronutrients, and vitamins (**11**), 50 mg/L myoinositol, 5 g/L KNO_3 , 20 g/L sucrose, pH 5.7.
2. M1: MSi medium plus 0.5 mg/L benzyl amino purine (BAP), 0.1 mg/L GA_3 and 8 g/L agar.
3. M1L: M1 medium without agar and growth regulators.
4. M2: MSi medium plus 0.1 mg/L BAP, 0.1 mg/L GA_3 , 7 g/L agar, 50 mg/L kanamycin, 250 mg/L cefotaxime.
5. M3: MSi medium plus 0.1 mg/L BAP, 7 g/L agar, 50 mg/L kanamycin, 250 mg/L cefotaxime.
6. BAP stock: dissolve 50 mg of 6-benzylaminopurine in 10 mL 1 N NaOH and make to 50 mL with water. Filter-sterilize and store in 1-mL aliquots at -20°C.
7. Gibberellic acid (GA_3) stock: dissolve 50 mg of gibberellic acid in 10 mL absolute ethanol and make to 50 mL with water. Filter-sterilize and store in 1-mL aliquots at -20°C.
8. Cefotaxime stock: dissolve 5 g of cefotaxime in 20 mL water. Filter-sterilize and store in 1-mL aliquots at -20°C.
9. Soil mix: 70% brunizem soil type (containing approximately 4% organic matter) and 30% turf.

3. Methods

3.1. Seed Disinfection

1. Place 50 seeds in 100-mL flasks and rinse for 2 min in sterile distilled water under vacuum to improve the seed-water contact. The next steps are conducted under the flow hood.
2. Sterilize seed with 70% EtOH for 2 min.
3. Wash with 4% bleach (2.25% active Cl) and 5 drops Tween-20. Place in desiccator under vacuum for 10 min and then stir on bench for 30 min.
4. Rinse five times (1 min each) with sterile distilled water.
5. Let seeds imbibe in sterile distilled water for 16 h (overnight) at 25°C in the dark.

3.2. Explant Preparation

1. Place the disinfected seeds on filter paper under the flow hood.
2. Take off the seminal peel including the internal thin membrane with the help of a scalpel and forceps under a binocular microscope.
3. Discard the radicle and the cotyledons by cutting them off at their base.
4. Using a surgical knife, cut through the middle of the meristem-containing explant into two identical pieces (Fig. 1). Each piece contains half of the apical meristem and the lateral cotyledonal meristems.

3.3. Agrobacterium Culture

1. Plate *A. tumefaciens* strain on YEP agar culture medium (with antibiotics) at 28°C for 2 d and maintain it at 4 to 8°C.
2. Transfer one colony of bacteria to 2 mL of MinA liquid culture medium (with antibiotics) and grow overnight at 28°C on an orbital shaker at 230 rpm.
3. Add 10 μ L acetosyringone solution (100 μ M final concentration) and shake the culture for 2 h before collecting the bacteria.
4. Collect the bacteria, when an exponentially growing stage is reached ($A_{660} = 0.6$), by centrifuging in 50-mL Corex tubes at 9000g for 15 min (see Note 3).
5. Wash the bacterial pellet twice with 2 mL of 10 mM $MgSO_4$.
6. Resuspend in 2 mL of 10 mM $MgSO_4$.

3.4. Cocultivation

1. Place 25 dissected explants in each Erlenmeyer flask containing 50 mL of M1 liquid medium and 0.830 mL of the resuspended bacteria (approx 1:60 dilution).
2. Cocultivate the explants with *Agrobacterium* for 72 h at 28°C in the dark with 120 rpm orbital agitation.

3.5. Selection Scheme and Regeneration of Transgenic Plants

1. At the end of cocultivation, remove *Agrobacterium* culture by passing the explants/culture through a funnel with sterile filter paper. Blot explants dry using filter papers.

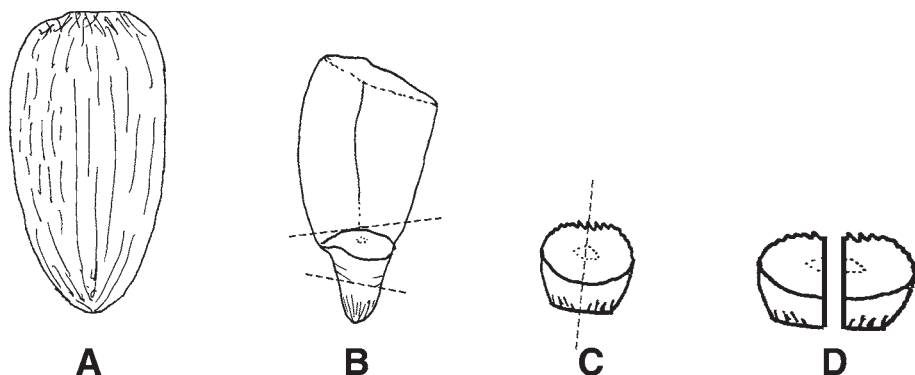


Fig. 1. Sunflower explant preparation scheme. **(A)** Disinfected achene (seed). **(B)** Mature embryo with the first cotyledon cut at the base. **(C)** Explant after cut of radicle, meristem, and both cotyledons. **(D)** Explant obtained, each carrying one-half of the apical meristem, ready to be treated with *Agrobacterium* and cultivated in vitro.

2. Place two infected explants with the cut side up in culture tubes containing M1 selective medium (M1 plus 50 mg/L kanamycin sulfate). Incubate tubes in growth chamber under a 12-h photoperiod at $23 \pm 2^\circ\text{C}$ (day) and $16 \pm 2^\circ\text{C}$ (night). Light condition: 2000 lux provided by daylight fluorescent tubes (see **Note 4**).
3. As a control for regeneration and selection efficiency, 30 explants (without *Agrobacterium* cocultivation treatment) are cultivated and placed on M1 medium with and without kanamycin.
4. Two weeks later, transfer explants to M2 medium. Subculture to fresh M2 medium once again after 15 d.
5. Depending on the shoot growth, it may take two to three subcultures on M2 medium. At this time, secondary shoots can be observed after the second subculture on M2 medium. If they are larger than 1 cm in height, they can be transferred to the M3 medium.
6. After the third subculture on M3 medium, if the shoots are larger than 2 cm in height and bright green and have vigorous growth, they can be transferred to greenhouse ($20 \pm 5^\circ\text{C}$; 16-h photoperiod; sunlight complemented with artificial light provided by mercury lamps; minimum 12,000 lux) for rustication and grafting (see **Note 5**).
7. Shoots from controls in selection medium should show chlorotic or bleaching leaves, low development, and no secondary shoots.

3.6. Grafting

1. To prevent early and consequently sterile flowering, shoots are grafted in the greenhouse (12). The best conditions for both rootstock and grafted plant growth are at 23°C , under a 16-h photoperiod and a minimum of 12,000 lux.

2. Primary transformant (T_0) plants from shoots that developed and survived on Km 50 medium for at least 45 d are transferred to the greenhouse to be acclimated and grafted.
3. Acclimatizing: after 3 d in the greenhouse, remove the lid of each tube 1 d before the grafting procedure.
4. The best rootstocks are commercial hybrid materials such as Contisol 9, developed from germinating seeds on 20-L pots containing fertile soil.
5. Rootstock plants that developed three to four leaves or 20 cm height are cut obliquely on the apical portion.
6. Immediately, each T_0 shoot is cut obliquely at the base and placed on the rootstock, making sure that conduction tissues of both parts are in close contact. The grafting area is tied up with a cotton strip and covered with a nylon bag to prevent dehydration.
7. During the next 5 d the bag must be progressively opened by making a 1-cm hole once a day. Grafting success rate can be 80 to 90%.

3.7. Flowering and T_1 Seed Harvesting

1. Each T_0 plant that survived the grafting procedure develops one or more flower heads. Compared with seed-borne plants, the capitulum size of the grafted plants can be quite small, but they usually have fertile disk flowers. Autopollination is desirable to give rise to achene development.
2. Seed T_1 fertility rate can vary between T_0 plants, from 30 to 90%, depending on the physiological state of the T_0 plants.
3. Molecular analysis (such as PCR and Southern) of transgenic plants can be carried out using leaf discs or petal tissues at the shoot regeneration or greenhouse stage.

4. Notes

1. To avoid germination problems, it is important to check out the seed viability when a new batch of seeds is used (number of seeds with germination capacity from 100 imbibed seeds).
2. Both *Agrobacterium* strains—C58 and EHA105—are efficient transformation agents using the protocol described. In our experience, C58 was successfully used to obtain several transgenic events using a binary plasmid system containing a cryA1 gene expressed under the 35S promoter control (10).
3. It is important to establish *Agrobacterium* growth curves (such as inoculum quantity needed for reaching desired OD) and conditions (such as shaker speed and incubator temperature) for each strain used for transformation.
4. Our experience suggests that initial explant number for infection should not be larger than 100, so the experiment can be performed by one operator. In case large-scale infection is conducted, involvement of more than one operator must be considered. The long hours of explant dissection may cause human fatigue that leads to decreased efficiency in plant transformation.

5. Shoots obtained from the first subcultures are most likely chimerical. Secondary shoots derived from the lateral buds that are obtained after the second subculture in the presence of selective media are considerably enriched in transgenic cells with the potential to develop transgenic flower tissues.

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V

LEGUME PLANTS

Alfalfa (*Medicago sativa* L.)

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Summary

A protocol for rapid, highly efficient transformation of alfalfa is described. Leaf explants from growth chamber-grown plants of a highly regenerable genotype are surface-sterilized, the margins are removed, and explants are inoculated with *Agrobacterium tumefaciens* strain LBA4404 carrying the T-DNA vector of interest. The explants and bacteria are cocultured for 7 to 8 d. Bacteria are removed by rinsing explants in sterile distilled water and by culture on regeneration medium containing the antibiotics carbenicillin or ticarcillin. Transformed callus is selected using kanamycin. Somatic embryos are induced by culture of callus on medium lacking plant growth regulators. As mature cotyledonary stage embryos arise, they are transferred to a fresh medium for shoot development and finally to a medium lacking kanamycin for continued shoot and root development. Transgenic plants can be produced in 9 wk with this protocol. Typically 60 to 80% of inoculated explants produce transgenic plants, and escapes are rare.

Key Words: Alfalfa; *Medicago sativa*; *Agrobacterium tumefaciens*; transformation; regeneration; cocultivation; somatic embryogenesis.

1. Introduction

Transformation of alfalfa has been achieved using a number of different methods (1). Leaf explant cocultivation with *Agrobacterium tumefaciens* followed by induction of somatic embryos is the most rapid and efficient method so far developed. In contrast to a number of legumes, alfalfa and several other *Medicago* species can be regenerated relatively easily via somatic embryogenesis and are thus amenable to transformation by *A. tumefaciens*. We describe a protocol that has been used to generate thousands of transgenic alfalfa plants for production of industrial enzymes (2–8), production of high-value compounds (9) enhancing tolerance to pathogens (10,11) and abiotic stress (12), and evaluating gene promoter activity (13–16).

The protocol utilizes highly regenerable genotypes selected from the variety Regen-SY (17). Although somatic embryogenesis has been observed in a number of varieties and germplasms (17–24), practically every plant from Regen-SY will regenerate rapidly under the conditions described. Use of other genotypes with the protocol may be successful, but chronology of the stages in regeneration and the efficiency of regeneration and transformation may not be as described below. The protocol utilizes *A. tumefaciens* strain LBA4404. Several other strains were tested and shown to be ineffective or to have lower overall transformation efficiencies (25). Other studies have also shown a strong strain-genotype interaction for transformation of alfalfa (23,24). Explants are derived from surface-sterilized leaves (trifoliolates) of clonally propagated growth chamber-grown plants. Periodically, source plants are initiated from plants propagated in vitro from shoot cuttings. Growth in soil can often result in plants obtaining endogenous bacterial and fungal contaminants. In vitro propagation ensures that clean stock materials are maintained. The cocultivation period recommended is 7 to 8 d. Shorter periods were evaluated, and transformation efficiency was found to be reduced (25). After removal of *A. tumefaciens* and plating on the selective medium, callus formation requires 2 to 3 wk. The only selective agent tested is kanamycin. Either carbenicillin or ticarcillin may be used to suppress *A. tumefaciens* growth. Explant-derived callus, often containing immature somatic embryos, is transferred to medium lacking plant growth regulators for further induction of somatic embryos and embryo development. A small amount of callus tissue will form on untransformed tissue under selection, but embryos do not form. Mature cotyledonary stage embryos form after 2 to 3 wk on this medium and are transferred to a medium for conversion to plantlets. After initiation of shoot growth, plantlets are transferred to a medium lacking kanamycin to stimulate root growth and for further shoot development. Plants may be transferred to soil after root formation. Transformed plants are obtained 9 to 14 wk after cocultivation.

On average, 60 to 80% of inoculated explants give rise to somatic embryos under selection. A very high number, typically 80 to 100% of the plants that develop from these embryos, contain the T-DNA.

2. Materials

2.1. Plant Materials

1. Alfalfa seed: this protocol is optimized for leaf explants from plants of the cultivar Regen-SY (17).

2.2. Agrobacterium Strains and Vectors

1. *A. tumefaciens* strain: this protocol is optimized for use of *A. tumefaciens* strain LBA4404 (26; see Note 1).

2. Binary vectors: this protocol is optimized for binary vectors carrying the *nptII* gene controlled by a constitutive promoter. Kanamycin is used for selection of transformed plants.

2.3. Culture Media and Stock Solutions

1. YEP medium (culture of *Agrobacterium*): 10 g/L protease peptone, 10 g/L yeast extract, 5 g/L NaCl. Mix components in double-distilled water and then autoclave in 250-mL aliquots for 20 min. For agar medium, add 15 g Bacto agar/L before autoclaving. Cool to 55°C before adding antibiotics (see **Note 2**).
2. Antibiotic stock solutions:
 - a. 25 mg/mL Rifampicin: dissolve rifampicin in DMSO, and store at -20°C in small aliquots.
 - b. 50 mg/mL Kanamycin: dissolve kanamycin in double-distilled water, filter-sterilize, and store in small aliquots at -20°C.

When using *A. tumefaciens* LBA4404, add 25 mg/L rifampicin plus the antibiotic specific to the transformation vector. For vectors with a kanamycin marker, add 50 mg/L kanamycin.

3. SH0 medium (holding medium for cut leaf tissue): Schenk and Hildebrandt basal salt mixture (Sigma), 1 mL/L 1000X Schenk and Hildebrandt vitamin solution (Sigma), 30 g/L sucrose, 0.5 g/L 2-(N-morpholino)ethanesulfonic acid (MES) dissolved in double-distilled water. Bring to pH 5.7 with 1 N KOH. Autoclave in 250-mL aliquots for 20 min.
4. For preparing SH0 medium without using prepackaged mixes, the macronutrients, micronutrients, and vitamins may be prepared and mixed as follows (use of myoinositol and plant growth regulators is optional): 100 mL/L SHII macronutrient stock, 1 mL/L SHIII micronutrient stock A, 1 mL/L SHIII micronutrient stock B, 5 mL/L SHIII iron stock, 10 mL/L SHIII vitamin stock, 30 g/L sucrose, 1.0 g/L myoinositol, 2 mg/L 2,4-D, 2 mg/L kinetin. Bring to pH 5.9 to 6.0 and then autoclave.
 - a. SHII macronutrient stock: 2 g/L $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$, 25 g/L KNO_3 , 4 g/L $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 3 g/L $\text{NH}_4\text{H}_2\text{PO}_4$.
 - b. SHIII micronutrient stock A: 5 g/L boric acid, 0.1 g/L $\text{CoCl}_2 \cdot 6 \text{H}_2\text{O}$, 0.2 g/L $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$, 10 g/L $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 1 g/L $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$.
 - c. SHIII micronutrient stock B: 1.0 g/L KI, 0.1 g/L $\text{Na}_2\text{MoO}_4 \cdot 2 \text{H}_2\text{O}$.
 - d. SHIII iron stock: 3 g/L $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$ and 4 g/L Na_2EDTA dissolved in hot distilled water.
 - e. SHIII vitamin stock: 0.5 g/L nicotinic acid, 0.5 g/L thiamine-HCL, 0.05 g/L pyridoxine-HCL.
5. Stock aminos (for 250 mL stock solution): 6.65 g glutamine, 0.83 g serine, 0.004 g adenine, 0.083 g L-glutathione in double-distilled water. Filter-sterilize and store at 4°C.

6. Preparation of growth regulators: dissolve 50 mg 2,4-D in 5 mL 100% ethanol and then bring to 50 mL with double-distilled water. Filter-sterilize and store at 4°C. To 10 mg kinetin add 0.1 mL 1 N NaOH to dissolve. Bring to 10 mL with double-distilled water. Filter-sterilize, aliquot, and freeze at -20°C.
7. B5h medium (cocultivation and callus initiation): 3.1 g/L Gamborg's B5 basal salt mixture (Sigma), 1.0 mL/L 1000X Gamborg's vitamin solution (Sigma), 0.5 g/L KNO₃, 0.25 g/L MgSO₄·7 H₂O, 0.5 g/L proline, 30 g/L sucrose in double-distilled water. Bring to pH 5.7 with 1 N KOH. Add 8 g/L Phytblend (Caisson Laboratories, Rexburg, ID). Autoclave in 500-mL aliquots for 20 min. Cool to approximately 55°C and add 30 mL/L stock aminos, 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.1 mg/L kinetin.
8. B5hKTc/C medium (selection of transformed tissue and removal of *Agrobacterium*): B5h medium with stock aminos and growth regulators plus 25 mg/L kanamycin and 500 mg/L ticarcillin or 250 mg/L carbenicillin (see **Note 3**).
9. B5h0KTc/C medium (induction and development of transgenic somatic embryos): B5h medium with stock aminos, plus 25 mg/L kanamycin and 500 mg/L ticarcillin or 250 mg/L carbenicillin. This medium lacks plant growth regulators.
10. MS0 medium (maintenance of in vitro plants): Murashige and Skoog basal salt mixture (Sigma) dissolved in double-distilled water, 30 g/L sucrose. Bring to pH 5.7 with 1 N KOH. Add 8 g/L Phytblend (Caisson Laboratories). Autoclave in 500-mL aliquots for 20 min.
11. MMS medium: Murashige and Skoog basal salt mixture (Sigma), 1 mL/L 1000X Nitsch and Nitsch vitamin solution (Sigma), 0.1 g/L myoinositol, 30 g/L sucrose in double-distilled water. Bring to pH to 5.7 with 1 N KOH, add 7.0 g/L Phytblend. Autoclave in 500-mL aliquots for 20 min.
12. MMSKTc/C medium (plant development medium): MMS medium with 25 mg/L kanamycin and 500 mg/L ticarcillin or 250 mg/L carbenicillin added after autoclaving.
13. MMSTc/C medium (rooting and plant development medium): same as MMSKTc/C medium but lacking kanamycin.

2.4. Tissue and Plant Culture

1. 100 × 15-mm sterile Petri plates.
2. 100 × 25-mm sterile Petri plates.
3. Autoclaved Whatman 3MM paper.
4. Micropore tape (cat. no. 5622-916; VWR International, West Chester, PA).
5. Shark skin filter paper (Schleicher and Schull, Keene, NH).
6. Culture tubes (22 mm) or Magenta GA7 vessels for culture of in vitro plants.
7. 8-inch Plastic pots.
8. 15-mL Glass test tubes for conditioning plantlets.
9. Cone-tainers (3.8 × 19 cm; Stuewe & Sons, Corvallis, OR) or small plastic pots.
10. Steam-pasteurized soil and sand (1:1, v/v).

3. Methods

3.1. Identifying Genotypes with a High Frequency of Regeneration

Plants with a capacity for regeneration in culture can be selected from a number of alfalfa varieties and germplasms (17–24). This protocol has been optimized for plants from the cultivar Regen-SY (17).

1. Surface-sterilize seed by submersing in 70% ethanol for 30 s.
2. Remove ethanol and place seed in a solution of 20% bleach with 0.1 to 0.05% Tween-20 for 10 min, agitating seed occasionally.
3. Rinse seed at least three times with sterile water.
4. Place individual seeds on MMS medium in Magenta GA7 vessels and incubate at 25°C under lights with a photoperiod of 16 h of 60 to 80 $\mu\text{E}/\text{m}^2/\text{s}$.
5. When plants are well grown (3–4 wk), carefully remove several leaves from each plant.
6. Cut each leaflet in half with a sterile scalpel blade, and place on B5h medium in a 100 $\times$ 25-mm Petri plate. Place plates in a growth chamber at 22 to 24°C with a 16-h photoperiod and light intensity of 60 to 80 $\mu\text{E}/\text{m}^2/\text{s}$.
7. After 3 wk, transfer callus to B5h medium without growth regulators to stimulate somatic embryo formation.
8. Embryos will form in 2 to 3 wk and may then be transferred to MMS for germination and conversion to plantlets. The original plant derived from an individual seed is the base material for stock plants maintained clonally in vitro.

3.2. In Vitro Plant Culture

1. Stock plants are maintained as sterile plants in vitro on MS0 medium in Magenta GA7 vessels or 22-mm culture tubes.
2. To subculture plants, remove shoot pieces with three to four nodes and insert the basal part of the stem approximately 0.5 cm into the medium. Seal lids with parafilm or micropore tape. Culture plants under lamps with a 16-h photoperiod of 60 to 80 $\mu\text{E}/\text{m}^2/\text{s}$ at 25°C. Plants may be maintained for up to 9 mo before subculturing or transplanting to soil.
3. To transplant to soil, remove plant from medium, trimming back long shoots, and plant in mixture of soil/sand (1:1; v/v) or soil-less potting mix such as Metro Mix 250 (W. R. Grace, Cambridge, MA).
4. Cover the pot with a plastic bag to maintain high humidity before placing the pot in a growth chamber with a 16-h photoperiod of 300 $\mu\text{E}/\text{m}^2/\text{s}$ and day and night temperatures of 21°C and 19°C, respectively. Remove the bag after 1 wk (see **Note 4**).

3.3. Plant Culture for Leaf Explants

1. Plants are grown in a growth chamber with a 16-h photoperiod of 300 $\mu\text{E}/\text{m}^2/\text{s}$ and day and night temperatures of 21°C and 19°C, respectively. To maintain vigorous growth, fertilize plants weekly with half-strength liquid complete fertilizer such as Peter's 10:10:10. Trim shoots to approximately 5 cm of the crown area every 3 to 4 wk (see **Note 5**).

3.4. *Agrobacterium tumefaciens* Culture

1. To initiate the *A. tumefaciens* culture, streak the culture onto a YEP agar plate with the appropriate antibiotics from a glycerol stock. Culture at 30°C for 2 to 4 d.
2. To initiate cultures for transformation, inoculate 3 mL YEP with antibiotics in a sterile 15-mL test tube with a single *A. tumefaciens* colony. Incubate at 28°C with shaking over night. Cultures of *A. tumefaciens* LBA4404 grown in YEP medium have a clumped appearance, which does not interfere with transformation. Use of the strain LBA4404 is recommended for high-efficiency transformation (see **Note 1**).

3.5. Transformation

1. Remove leaves from nodes two to five (unexpanded leaf is node one) of growth chamber-grown plants. The best leaves are large, thick, dark green, and very healthy looking. Do not use leaves from flowering plants. Submerge leaves in cool tap water in a 50-mL test tube or float leaves on cool tap water in a deep Petri dish or beaker.
2. Working in a laminar flow or biosafety hood, surface-sterilize leaves (about six leaves per batch) in 70% ethanol for 5 to 10 s, just enough to wet leaves. Transfer leaves to 10 to 20% bleach with 0.1 to 0.05% Tween-20 for 1.5 min. Rinse at least three times with sterile water. It is easiest to set up a series of sterile 100 × 25-mm Petri dishes with each solution and move leaves sequentially into each solution.
3. Remove leaf from third rinse water. Place leaf in a sterile Petri dish or on moist shark skin filter paper and with a sharp scalpel blade separate leaflets, remove leaflet margins, and cut the leaflet in half cross-wise so that pieces are about 0.5 × 0.5 cm. Immediately place leaf pieces in 12 mL SH0 medium in a sterile 50-mL test tube. For a typical experiment, prepare approximately 72 explants (12 trifoliate leaves).
4. After all explants have been prepared, add 3 mL of the overnight *A. tumefaciens* culture to the explants. Alternatively, when sufficient explants have been prepared, they are moved to a suspension of *A. tumefaciens* cells from an overnight (or 2-d) culture grown in YEP selection medium. Cell density is adjusted with YEP to fall between 0.6 and 0.8 at A₆₀₀ nm. Remove leaves from the *A. tumefaciens* solution after 15 to 30 min and blot them briefly on sterile filter paper to remove excess liquid.
5. Place inoculated explants on B5h medium in 100 × 15-mm Petri plates, approximately 12 explants/plate. Move the explants carefully and avoid excessive bruising of the tissue. Seal the plates with micropore tape and place in a 24°C incubator with light intensity of 30 μE/m²/s (place a piece of cheesecloth over plates in a chamber at 60–80 μE/m²/s) and 16-h photoperiod.
6. Seven days after inoculation, remove leaf pieces from plates, place in 25 mL sterile distilled water in a sterile 50-mL test tube, and invert 10 to 20 times to remove bacteria. Pour off water and replace with fresh sterile distilled water.

Repeat the rinsing procedure two or three times. Blot leaf pieces briefly on sterile filter paper and place on B5hKT/C medium in 100 × 25-mm Petri dishes, approximately 45 mL/plate. Place only 10 to 12 explants/plate as explants will enlarge considerably (see **Note 6**).

7. Plates are maintained in the growth chamber at 22 to 24°C, with a 16-h photoperiod and light intensity of 60 to 80 $\mu\text{E}/\text{m}^2/\text{s}$ for 2 to 3 wk. Callus formation occurs rapidly at this time, and the original explant may no longer be intact at the end of this period. A mock inoculation treatment (no *Agrobacterium*) is highly recommended for each experiment. Place explants on B5hKTc/C medium to test for kanamycin selection. Place explants on B5h medium to test for regeneration.

3.6. Somatic Embryogenesis and Plant Regeneration

1. Transfer explants and associated callus cells to B5h0KTc/C medium in 100 × 25-mm Petri plates. Return plates to a growth chamber at 22 to 24°C, with a 16-h photoperiod and light intensity of 60 to 80 $\mu\text{E}/\text{m}^2/\text{s}$.
2. Over the next 3 wk somatic embryos will form. These first appear as small dark green buds embedded in callus, enlarge to torpedo-shaped embryos, and finally form cotyledons, which may be fused. Pale green or white embryos, often malformed, are likely to be escapes. Transfer only dark green, mature embryos to MMSKTc/C medium, carefully placing the embryo upright with approximately one-fourth of the embryo embedded in the medium. If embryos have formed a clump and cannot be separated, transfer the clump to the medium and as the embryos continue to develop they can be separated. Each explant piece can produce 10 to 50 embryos. These may or may not be independent events (see **Note 7**) such that it is more expedient to generate a population of plants for analysis with each plant derived from a different explant piece. It is still advisable to culture at least 10 embryos from each callus piece at this stage because not all will convert to plants. Number each explant and keep separate the embryos from each explant.
3. Over the next 1 to 3 wk the embryos will form a shoot and sometimes a root. Rooting is inhibited by kanamycin. Move green plantlets to MMSTc/C medium in a Magenta GA7 vessel, approximately 60 mL/vessel, for further shoot and root development. Approximately nine plantlets will fit in a Magenta GA7 vessel. Not all embryos will convert to a plantlet.
4. After most plants in a vessel have formed roots, remove rooted plants and place individually in 15-mL test tubes filled with water. Submerge all but the top third of the plant in the water and let stand on the laboratory bench (approximately 25°C, ambient light) for 2 to 4 d to condition plantlets. Some drying of leaflet margins will occur.
5. Transplant into soil/sand (1:1; v/v) in cone-tainers or small pots, water well, and place in a growth chamber with a 16-h photoperiod of 300 $\mu\text{E}/\text{m}^2/\text{s}$ and day and night temperatures of 21°C and 19°C, respectively. Apply half-strength complete fertilizer such as Peter's 10:10:10 after 7 d of growth.

3.7. Plant Propagation

1. Alfalfa can be propagated easily from stem cuttings (ramets). Excise stem sections with one to three nodes using a sharp razor blade and place the base into moist vermiculite, sand, or potting mix in the growth chamber or greenhouse. Adventitious roots will form in approximately 7 to 10 d.
2. Alfalfa is typically an outcrossing species with strong inbreeding depression. Although many alfalfa plants will form seed after self-pollination by hand-tripping flowers (27), progeny can have low vigor. Cross-pollination with a wild-type plant is recommended for producing seed from transgenic alfalfa (1,28).

4. Notes

1. Use of *A. tumefaciens* strain LBA4404 as the transforming strain is critical for efficient transformation. Other strains may cause explant browning and low efficiency of transformation (25).
2. All agar media may be prepared in 500-mL aliquots and autoclaved in 1-L bottles. Media may be prepared up to 1 wk in advance and melted in a microwave. Amendments such as growth regulators and antibiotics should be added after melting and cooling to 55°C.
3. Ticarcillin (SmithKline Beecham Pharmaceuticals, Philadelphia, PA) is usually available from veterinary pharmacies on university campuses or through distributors of plant tissue culture media. It is generally less expensive than carbenicillin, has high quality control, and is packaged as a sterile powder. Both ticarcillin and carbenicillin are dissolved in sterile water and stored in small aliquots at -20°C.
4. Growth chamber-grown plants provide much larger and more resilient explants than in vitro grown plants and require less labor to produce and maintain. Leaves from in vitro grown plants are not recommended for this protocol.
5. Alfalfa plants are a host for thrips that cause white spots on leaves and leaf deformation. Use of insecticides may impact tissue culture and should be infrequent. Maintaining a clean growth chamber will reduce thrip infestations. Some alfalfa varieties such as Regen-SY are susceptible to a wilt disease caused by *Acremonium* sp. If treated at the first sign of wilting with the fungicide Benomyl, plants can recover.
6. If growth of *Agrobacterium* is excessive after 4 d (explants completely covered with bacteria and a halo of bacteria around each explant), it can be advantageous to rinse the explants twice in sterile distilled water at this stage and place them on fresh medium for a further 4 d of cocultivation.
7. Alfalfa somatic embryos were shown to arise from a single cell (29); however, secondary embryos can form (18). Thus, it is important to track regenerated plants to ensure that each transformant analyzed is an independent transformation event.

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Chickpea (*Cicer arietinum* L.)

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Summary

Chickpea is one of the most important leguminous, cool-season, food crops, cultivated prevalently in the Asian Pacific region. In spite of its nutritional importance, its area of cultivation has been low, with virtually no increase. Conventional breeding has resulted in several important improvements in this crop, and recent advances in biotechnology such as plant tissue culture and genetic transformation can significantly contribute to better sustainability of this important food crop. Here, we describe an efficient *Agrobacterium*-mediated transformation protocol for chickpea using axillary meristem explants, which results in a high frequency of genetic transformation (70%) and recovery of valuable transgenic plants. The protocol is significant owing to its high reproducibility and recovery of the transgenics in a relatively short period (90–100 days).

Key Words: Chickpea; *Cicer arietinum*; genetic transformation; hardening; shoot regeneration; tissue culture, rooting of shoots; transgenic plants.

1. Introduction

Chickpea (*Cicer arietinum* L.) is one of the important grain legumes that play a significant role in the nutrition of the rural and urban poor in the developing world. Chickpea is traditionally grown in many parts of the world, including Asia, Africa, Europe, and North and South America, and it contributes 15% to the world pulse harvest of about 58 million tons annually. Despite significant gains in world pulse production during the last two decades (annual growth rate of 1.9%), chickpea production growth has been slow. This slower pace in chickpea production has been the result of various refractory biotic and abiotic constraints such as ascochyta blight (AB), botrytis grey mold (BGM), dry root rot, collar rot, fusarium wilt, pod borer, and abiotic stress like drought and low temperature. The available chickpea germplasm lacks effective resistance for use in developing insect pest-resistant genotypes.

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Classical and modern breeding technologies have resulted in limited success in interchange of the desirable characters in this important pulse crop. However, biotechnological techniques have emerged as a potential supplement to these efforts. Advances in plant tissue culture and genetic transformation methodologies have paved the way for alternative crop improvement and creation of an elite germplasm. However, reliable regeneration and transformation protocols have not emerged, owing to the perceived recalcitrant nature of chickpea toward tissue culture. Although several regeneration and transformation protocols involving somatic embryogenesis and organogenesis have been reported with varying success rates (1–7), effective chickpea regeneration has been possible only through use of explants based on cotyledonary nodes or shoot apices derived from seedling explants (3). Asynchronous shoot bud production makes a number of chickpea regeneration systems inefficient for genetic transformation (8–10). In addition, rooting and transplanting of the *in vitro* recovered chickpea plants have remained a major bottleneck in the application of transformation technology for serious crop improvement programs.

A prerequisite for the *in vitro* manipulation of a plant species is the availability of an efficient and reproducible plant regeneration system. In chickpea, several shoot regeneration protocols have been reported (3). However, low success rates for recovery of plants make these protocols inefficient for genetic transformation. The protocol detailed here has been optimized using various tissue culture variables in pursuit of an efficient and reproducible transformation and regeneration procedure based on regeneration procedures described earlier (11) for high-frequency genetic transformation (Sharma et al., unpublished results) and recovery of valuable transgenic chickpea plants. The method involves the use of the axillary meristem explant produced by removing the axillary bud and overcoming the apical dominance of the shoot buds. Results are better in terms of regeneration and transformation efficiency. T_0 generation of the putative transformants tested for incorporated genes by using polymerase chain reaction (PCR) and Southern hybridization techniques showed a transformation frequency of 70%. The transformation efficiency is defined as the percentage of PCR-positive independent events from 100 putative events generated following antibiotic selection.

2. Materials

2.1. Plant Material and Sterilization

1. Chickpea seeds of the variety C-235 were obtained from the gene bank of the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT).
2. 70% Ethanol.
3. 0.1% (w/v) Aqueous mercuric chloride.
4. Sterile double-distilled water.

2.2. Plant Tissue Culture Stock Solutions and Media

1. Murashige and Skoog's medium (MS): MS basal components (12), 3% sucrose, pH adjusted to 5.8 prior to autoclaving.
 - a. Major salt (50X): prepare stock solutions of the major salts of MS medium as follows:
 - i. NH_4NO_3 : dissolve 33 g of the chemical in 200 mL of sterile distilled water. Store the stock at room temperature for no more than 1 mo. Use 10 mL of the stock solution for preparing 1 L of the medium.
 - ii. KNO_3 : dissolve 38 g of the chemical in 400 mL of sterile distilled water. Store the stock at room temperature for no more than 1 mo. Use 20 mL of the stock solution for preparing 1 L of the medium.
 - iii. KH_2PO_4 : dissolve 3.40 g of the chemical in 200 mL of sterile distilled water. Store the stock at room temperature for no more than 1 mo. Use 10 mL of the stock solution for preparing 1 L of the medium
 - iv. CaCl_2 : dissolve 8.80 g of the chemical in 200 mL of sterile distilled water. Store the stock at room temperature for no more than 1 mo. Use 10 mL of the stock solution for preparing 1 L of the medium
 - v. $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$: dissolve 7.40 g of the chemical in 200 mL of sterile distilled water. Store the stock at room temperature for no more than 1 mo. Use 10 mL of the stock solution for preparing 1 L of the medium
 - b. Minor salts (100X): weigh the required quantities of the minor salts (83 mg KI, 2230 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 860 mg $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$, 25 mg $\text{Na}_2\text{MoO}_4 \cdot 2 \text{H}_2\text{O}$, 2.5 mg $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$, 2.5 mg $\text{CoCl}_2 \cdot 6 \text{H}_2\text{O}$) and dissolve in 100 mL of sterile distilled water. Store the stock 4°C for no more than 1 mo. Use 5 mL of the stock solution for preparing 1 L of the medium
 - c. Iron (100 X):
 - i. $\text{Na}_2\text{EDTA} \cdot 2 \text{H}_2\text{O}$: dissolve 3.73 g of the chemical in 1000 mL of sterile distilled water. Store the stock at 4°C for no more than 1 mo. Use 5 mL of the stock solution for preparing 1 L of the medium.
 - ii. $\text{FeSO}_4 \cdot 7 \text{H}_2\text{O}$: dissolve 2.78 g of the chemical in 1000 mL of sterile distilled water. Store the stock at 4°C for no more than 1 mo. Use 5 mL of the stock solution for preparing 1 L of the medium.

OR

- iii. FeNa_2EDTA : dissolve 2 g of the chemical in 500 mL of sterile distilled water. Store the stock at 4°C for no more than 1 mo. Use 5 mL of the stock solution for preparing 1 L of the medium.
2. Kinetin (Sigma): 1 mM stock. Dissolve the 21.5 mg of hormone powder in a few drops of 1 M HCl and make up the final volume using sterile distilled water. Store the stock at -20°C .
3. Indole-3-butyric acid (IBA; Sigma): 1 mM stock. Dissolve 20.3 mg of the powder in a few drops of ethanol and adjust the final volume using sterile distilled water. Store at -20°C for up to 3 mo.

4. 2-Isopentenyladenine (2-iP): 1 mM stock. Dissolve 20.32 mg of the chemical in a few drops of 1 N NaOH and adjust the final volume by using sterile distilled water. Store at -20°C for up to 3 mo.
5. Thidiazuron (TDZ): 1 mM stock. Dissolve 22.25 mg of the powder in 100 mL of DMSO. Store at -20°C for up to 3 mo.
6. Giberellic acid (GA_3): 1 mM stock. Dissolve 34.6 mg of the powder in 100 mL of water. Store at -20°C for up to 3 mo.
7. Shoot induction medium (SIM): MS medium, 4 μM TDZ, 10 μM 2-iP, 2 μM kinetin (adjust pH to 5.8), 0.8% agar for solidification.
8. Shoot elongation medium 1 (SEM1): MS medium, 5 μM 2-iP, 2 μM kinetin (adjust pH to 5.8), 0.8% agar.
9. Shoot induction medium 2 (SEM2): MS medium, 2 μM GA_3 (adjust pH to 5.8), 0.8% agar.
10. Cefotaxime: 125 mg/mL stock. Dissolve the powdered chemical in water and filter-sterilize the solution prior to use.
11. Kanamycin monosulfate (Sigma): 125 mg/mL stock. Dissolve the kanamycin in water. Filter-sterilize the stock and store in aliquots at -20°C for no more than 15 d.

2.3. Bacterial Culture

1. *Agrobacterium* strain and vector: disarmed *Agrobacterium tumefaciens* strain C58, harboring binary plasmids pBI121 with *nptII* as a selectable marker gene.
2. Luria-Bertani medium (LB): 1% Bacto-tryptone, 0.5% Bacto yeast extract, 1% NaCl, 1.5% agar. Adjust the pH to 7.0 with 5 N NaOH (approx 0.2 mL) prior to autoclaving.
3. Yeast extract medium (YEB): 0.5% Bacto-peptone, 0.1% yeast extract, 0.5% beef extract, 0.5% sucrose, 0.05% $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$. Adjust pH to 7.0 with NaOH prior to autoclaving.

2.4. Root Induction and Hardening Conditions

1. Root induction medium (RIM): modified MS medium with 9.4 mM of KNO_3 , 5 μM IBA. Adjust the pH to 6.0.
2. Hydroponics system: $\frac{1}{2}$ strength Arnon's nutrient solution, pH 6.5 (13), 3 μM IBA in an 8-cm Magenta jar. Arnon's nutrient solution is composed of four different stock solutions of the major and minor salts.
 - a. Stock I: 12.2 g of KH_2PO_4 , 15.5 g of KCl, 25 g of $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$. Dissolve the salts in 1000 mL sterile distilled water. Store the solution at room temperature for no more than 1 mo. Use 10 mL of this stock for preparing 1 L of the nutrient solution.
 - b. Stock II: 21.5 g of $\text{CaCl}_2 \cdot 2 \text{H}_2\text{O}$ or 25 g of $\text{CaSO}_4 \cdot 2 \text{H}_2\text{O}$. Dissolve the salts in 1000 mL sterile distilled water. Store the solution at room temperature for no more than 1 mo. Use 10 mL of this stock for preparing 1 L of the nutrient solution.
 - c. Stock III: 1 g of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.25 g of $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.25 g of $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$, 0.05 g of $\text{Na}_2\text{MoO}_4 \cdot 2 \text{H}_2\text{O}$. Dissolve the salts in 1000 mL sterile distilled water.

Store the solution at room temperature for no more than 1 mo. Use 1 mL of this stock for preparing 1 L of the nutrient solution.

- d. Stock IV: 30 g of $\text{FeC}_6\text{H}_5\text{O}_7 \cdot 5 \text{H}_2\text{O}$ or 15 g of FeCl_3 or 59 g of NaFe-EDTA. Dissolve the salts in 1000 mL sterile distilled water. Store the solution at room temperature for no more than 1 mo. Use 1 mL of this stock for preparing 1 L of the nutrient solution.

2.5. Transplantation

1. Pots: 8- and 20-cm-diameter pots.
2. Fungicides: Thiram® and Bavistin®.
3. Polypropylene bags.
4. Potting mixture: smooth and coarse sand and black farm soil (3:2), 10% organic Cell Rich® and rice straw compost (1:1).

3. Methods

3.1. Plant Material and Preparation of Explant

1. Healthy and mature seeds of chickpea variety C-235, a widely grown cultivar, is used as starting material for subsequent procedures.
2. Surface-sterilize the seeds with 70% (v/v) ethanol for 1 min, followed by treating with 0.1% mercuric chloride for 10 min. (Unless otherwise noted, all procedures are carried out under aseptic conditions.)
3. Rinse the seeds two to three times with sterile distilled water prior to soaking overnight.
4. Decoat the soaked seeds and place the seeds for germination on SIM at a density of 10 to 15 seeds per plate. Seal the plates with Parafilm. Allow the seedlings to grow at $26 \pm 1^\circ\text{C}$ under continuous light conditions ($60 \mu\text{E}/\text{m}^2/\text{s}$ light intensity provided by cool white fluorescent lamps) for 5 to 7 d until the axillary buds are prominent.
5. Carefully remove the axillary buds up to the base and make two cuts through the axillary meristem in order to remove the shoot and root tips up to the hypocotyl and epicotyl regions, respectively (Figs. 1 and 2A).
6. Subculture the axillary meristem explants (AMEs) thus obtained on a plate containing SIM for another 7 d at $26 \pm 1^\circ\text{C}$ under continuous cool white light provided by fluorescent lamps ($60 \mu\text{E}/\text{m}^2/\text{s}$).
7. Carefully remove the emerging shoot buds from the enlarged base of the axillary bud (Figs. 1 and 2B) by scraping with a sharp scalpel blade to obtain the explant (AM4) for transformation purposes (Figs. 1 and 2C).

3.2. Agrobacterium Culture Preparation and Explant Infection

1. Inoculate a single colony of *A. tumefaciens* strain C58 harboring the gene of interest in 25 mL YEB liquid medium containing appropriate antibiotics and allow to grow overnight at 28°C on an incubator-shaker.
2. Ensure that the OD of the overnight grown culture is between 0.6 and 1.0.
3. Divide the culture into two (12 mL culture per tube) in 25-mL centrifuge tubes. Centrifuge at 600g for 5 min.

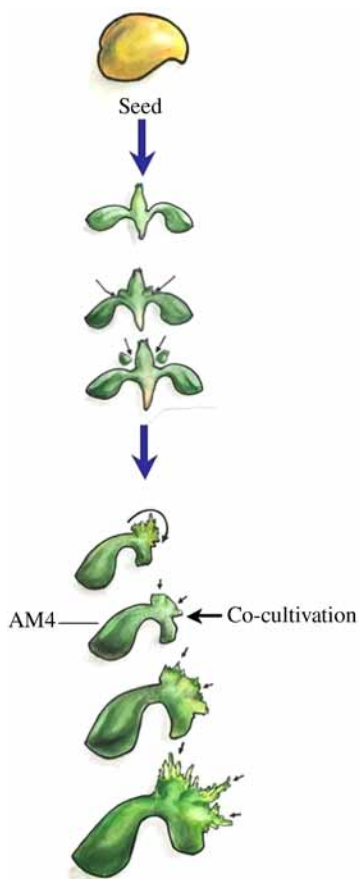


Fig. 1. Diagrammatic representation of the preparation of axillary meristem explants (AM4) of chickpea for *Agrobacterium*-mediated genetic transformation. Large arrows depict the progression of explant preparation. Medium and circular arrows indicate sites of surgery, and small arrows show sites of multiple shoot regeneration.

4. Discard the supernatant carefully and wash the pellet with 10 mL of sterile $\frac{1}{2}$ strength MS medium.
5. Centrifuge the cells at 600g for 2 to 3 min to collect the cell pellet.
6. Resuspend the pellet in 25 mL of sterile $\frac{1}{2}$ MS and pour in a sterile Petri plate for infecting the prepared explants.
7. Briefly dip the explants (AM4; **Figs. 1** and **2C**) in the *Agrobacterium* culture for 1 to 2 s and culture five to seven explants on SIM with the base of the cotyledon embedded in the medium.
8. Cocultivate the explants with the bacteria for 48 h, at $26 \pm 1^\circ\text{C}$ under continuous cool white light provided by fluorescent lamps ($60 \mu\text{E}/\text{m}^2/\text{s}$).

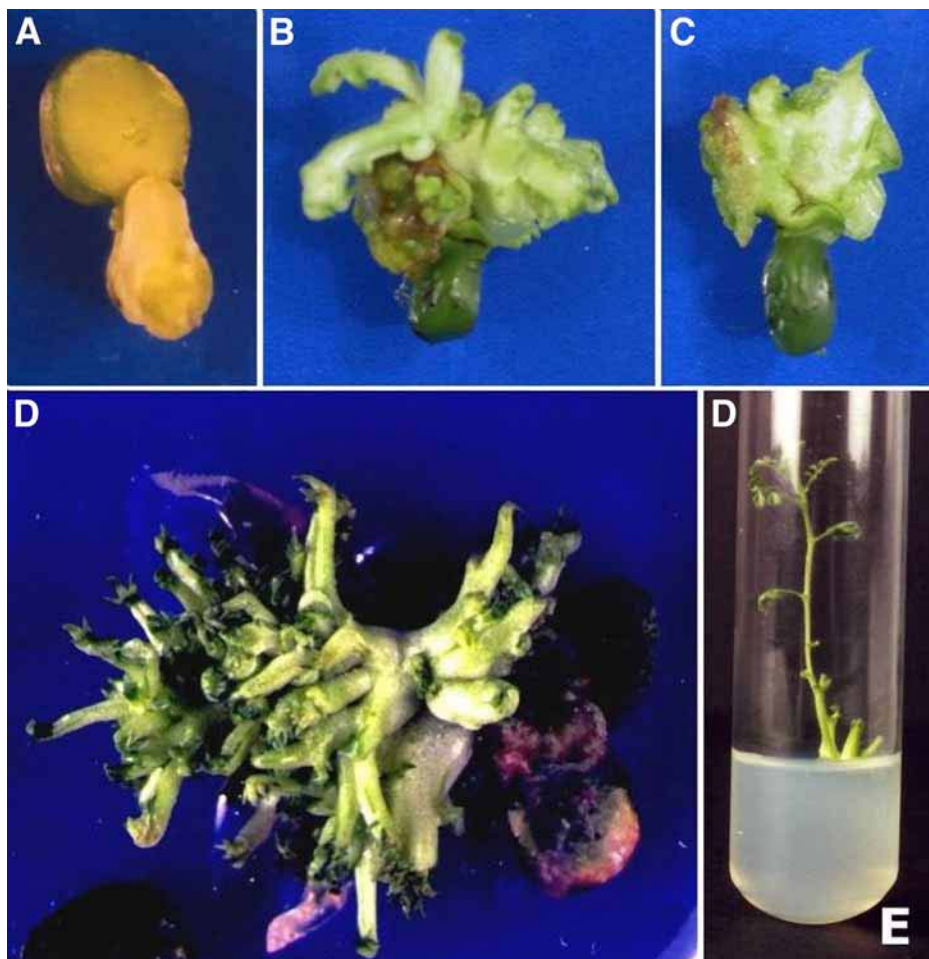


Fig. 2. Regeneration of adventitious shoot buds and plants from axillary meristem explants of chickpea. (A) Axillary meristem explant derived from presoaked chickpea seeds. (B) Regeneration of multiple shoot buds from AME after 5 d of culture on SIM. (C) Explant containing swollen area after removal of regenerating shoot buds (AM4) after 7 d of culture on SIM. (D) Multiple shoots originating from different parts of regenerating area after 12 to 14 d of culture on MS. (E) Elongation of shoots on SEM after 1 to 2 wk of culture.

3.3. Regeneration and Multiplication of Shoots

1. After cocultivation, transfer the explants to MS medium containing 250 mg/L cefotaxime for 4 to 5 d at $26 \pm 1^\circ\text{C}$ under continuous cool white light provided by fluorescent lamps ($60 \mu\text{E}/\text{m}^2/\text{s}$).

2. Transfer the explants onto MS medium containing 250 mg/L cefotaxime and a low selection pressure (e.g., 25 mg/L kanamycin or 2 mg/L hygromycin) for 1 wk under the same culture conditions (see **Note 1**).
3. Subculture the explants containing multiple shoot buds (**Fig. 2D**) on MS medium containing a relatively higher selection pressure (50 mg/L kanamycin or 5 mg/L hygromycin) for 7 to 10 d (see **Note 2**).
4. Carefully separate the bunches of emerging shoot buds from the cotyledon part with some intact basal callus and transfer to shoot elongation medium (SEM1) containing a higher selection pressure (75 mg/L kanamycin or 7.5 mg/L hygromycin) for another 10 d (**Fig. 2C**).
5. Transfer the elongated (**Fig. 2E**) as well as unelongated shoots to SEM2 containing a stringent selection pressure (100 mg/L kanamycin or 10 mg/L hygromycin) for two to three passages at 7-d intervals.
6. Carefully separate the healthy growing shoots from the elongating shoot bunch, and remove the untransformed bleached shoots.
7. Replace the stunted shoots on SEM2 for two to three passages of 1 wk each for further elongation and increasing the length of internodes (see **Note 3**).

3.4. Rooting of Shoots

1. Phase 1: select dark green, healthy shoots approx 5 cm long for induction of adventitious roots. Cut the basal stem segment of the elongated shoot in half so it does not contain any nodal meristem, and culture it on a filter paper bridge immersed in root induction medium (RIM) for 1 to 2 wk (**Fig. 3A**; see **Notes 4–6**).
2. Phase 2: pulse treat the shoots that fail to form roots within 1 to 2 wk by dipping the shoots in 100 μ M IBA followed by culturing on filter papers immersed in the liquid MS medium.
3. Phase 3: use a hydroponic system for inducing roots in the shoots that do not root even after two to three subcultures on RIM. Fill 8-cm-diameter Magenta jars with $\frac{1}{2}$ strength Arnon's solution containing 3 μ M IBA and suspend the shoot with a support such that 1 cm of the shoot base is immersed in the solution (**Fig. 3B**). Change the media after every 4 to 5 d until the root primordia appears. Transfer the shoots with roots to hormone-free Arnon's solution for further growth and development.

3.5. Hardening and Transplantation of Rooted Shoots

1. Take the plants out of the tubes carefully, wash the roots thoroughly, dip in 0.5% Thiram solution, and transfer each of the rooted shoots to 8-cm-diameter pots containing 2 to 4 mm of sand (**Fig. 3C**). Water the pot optimally, cover the plants completely with polypropylene bags, and gradually open the covers over 7- to 10-d period (see **Note 7**).
2. Transfer the hardened plants to 20-cm-diameter pots containing the potting mixture.

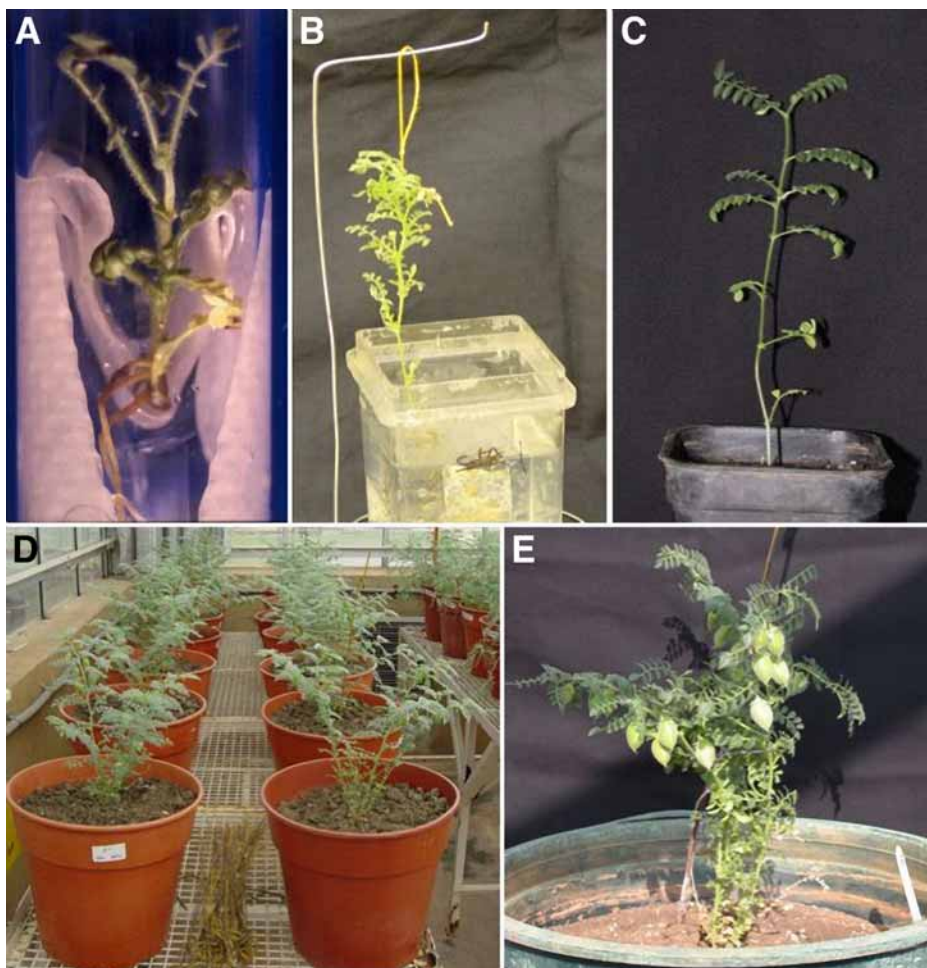


Fig. 3. Rooting, hardening, and transplantation of in vitro regenerated chickpea plants. **(A)** Rooting of elongated shoot on a filter paper bridge soaked in RIM. **(B)** Hardening of rooted plantlets in a hydroponic system. **(C)** Transplanted chickpea plant after hardening for 10 to 12 d as in (B). **(D)** Hardened and transplanted chickpea plants growing in greenhouse conditions. **(E)** Mature chickpea plant bearing pods containing viable seeds at the time of harvest after 100 d of transplantation.

3.6. Maintenance of Plants in the Greenhouse and Seed Production

1. Transfer the plants to a containment greenhouse with 24/18°C day/night temperatures and allow these to grow (**Fig. 3D**) until maturity and subsequent progression of generations (T_1 , T_2 , and so on). Normally it takes approx 100 d from transplantation for the plants to mature for harvesting (**Fig. 3E**).

2. Remove the terminal buds of some branches and remove the emerging flowers to extend the vegetative growth of the plant.
3. Harvest the seeds as soon as the pods turn yellow to yellowish brown in color; this can also be postponed until the mother plant is completely dry. The typical seed set rate is approx 45 to 55 seeds per plant.

3.7. Characterization of Putative Transformants

1. Carry out extraction of genomic DNA using young leaflets of the putative transformants following the protocol of Dellaporta et al. (14). Typically 1 g of fresh young leaflets results in approx 50 to 60 µg of genomic DNA.
2. Use approx 200 ng of purified DNA for a PCR amplification reaction using standard procedures followed by assay of PCR products on 1.2% agarose gels.
3. For Southern blot hybridization, we typically use 10 to 15 µg of total DNA and digest it with appropriate restriction enzymes that cut only once on T-DNA. The digested DNAs are resolved on 0.8% agarose gel, blotted onto a nylon membrane, and hybridized with gene-specific probes (radioactive or nonradioactive) following the standard protocol (15).
4. Carry out genetic analysis to ascertain the inheritance pattern of the introduced genes in the T₁ generation progenies of primary transformants. This is done by growing all the progenies of the selected events (primary positively confirmed transformants) and then carrying out PCR analysis on the genomic DNA for the introduced genes. The segregation of a single-copy insert usually follows a Mendelian inheritance pattern of 3:1 (i.e., 3 positives to 1 negative progeny). In the case of chickpea, selection of the seedlings on kanamycin- or hygromycin-containing medium is not very reliable.

4. Notes

1. A progressive selection system with stepwise increases in the concentration of the selective agent at each stage has been found to be useful in obtaining stringently selected putative transformants in chickpea.
2. The bleached shoots must be carefully removed at each stage to prevent escapes.
3. Subculture of stunted shoots on SEM2 for one to two extra passages increases the frequency of elongation and the length of the internodes.
4. Imposition of any selection pressure must be avoided at the rooting stage as it decreases the rooting frequency.
5. Inclusion of any nodal meristem on the surface exposed to rooting medium drastically reduces the rooting frequency of the shoots.
6. Use of filter paper bridges for rooting is beneficial, as it prevents the shoots from desiccating, resulting in efficient rooting of the shoots.
7. The rooted shoots should ideally be transferred to the hardening phase within 10 d of the root primordia being observed.

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Clovers (*Trifolium* spp.)

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Summary

Legumes constitute one of the most important global groups of agricultural species, providing a major source of protein and oil for humans and animals as well as fixing nitrogen and improving the fertility of soils. Gene technology can assist plant improvement efforts in clovers (*Trifolium* spp.), aiming to improve forage quality, yield, and adaptation to biotic and abiotic stresses. An efficient and reproducible protocol for *Agrobacterium*-mediated transformation of a range of *Trifolium* species, using cotyledonary explants and different selectable marker genes, is described. The protocol is robust and allows for genotype-independent transformation of clovers. Stable meiotic transmission of transgenes has been demonstrated for selected transgenic clovers carrying single T-DNA inserts recovered from *Agrobacterium*-mediated transformation. This methodology can also be successfully used for 'isogenic transformation' in clovers: the generation of otherwise identical plants with and without the transgene from the two cotyledons of a single seed.

Key Words: *Agrobacterium tumefaciens*; transgenic plants; *Trifolium* species (clovers); cotyledonary explants; gene technology.

1. Introduction

Legumes are important for sustainable agriculture because of their nitrogen-fixing capacity and high nutritive value in production systems based on grazing animals. Legumes produce many complex natural products, which may have evolved to mediate the establishment of symbiosis and to protect plants against abiotic and biotic stresses. These products include a large spectrum of flavonoids, isoflavonoids and anthocyanins, condensed tannins, and triterpene saponins. Modification of the level and composition of some of these compounds using gene technology may lead to forage cultivars with improved nutritional qualities and tolerance of biotic and abiotic stress. Low levels of

condensed tannins (1–3% by dry mass) in forage can improve protein uptake by ruminants and reduce the risk of pasture bloating by slowing ruminal degradation of plant proteins and increasing crude protein flow to the intestine (1,2). Modification of the phenylpropanoid pathway in legumes could potentially lead to ‘bloat-safe’ cultivars with an elevated level and improved composition of condensed tannins in leaves.

Gene technology can further contribute to the molecular breeding of legumes for virus resistance. Some clovers (*Trifolium* spp.) that are an important component of temperate pastures throughout the world are susceptible to infection by many viruses, such as alfalfa mosaic virus (AMV), white clover mosaic virus (WCMV), and clover yellow vein virus (CYVV). Infection with these viruses can reduce potential biomass production in white clover-dominated pastures by up to 30% (3–6). Although potential sources of virus resistance or tolerance have been described in some legumes (7,8), introgression of genes for resistance to AMV and WCMV into white clover dependent on conventional breeding methods has not been successful. However, AMV resistance has been demonstrated in transgenic white clover plants expressing chimeric AMV coat protein genes (9,10).

Genetic modification of forage legumes requires robust and efficient methods for genetic transformation and plant regeneration that are largely genotype independent. Transformation of *Trifolium* species with the aim of modifying agronomically important traits has been reported (11–15). Strategies for the regeneration of pasture legumes include organogenesis and somatic embryogenesis using different sources of tissue explants, such as stolons, immature embryos, cell suspension cultures, and protoplasts (16–19). Generally, elite cultivars of most forage legumes do not show high levels of regeneration (20).

The protocol described here is applicable to different *Trifolium* species such as white clover (*Trifolium repens*, cvs. Haifa, Huia, Irrigation, Sustain, and Mink); red clover (*T. pratense* cvs. Astred, Colenso, Cherokee, Quinequeli, Redquin, and Renegade); subterranean clover (*T. subterraneum* subsp. *brachycalycinum* cv. Clare, subsp. *subterraneum* cvs. Denmark and Woogenellup, and subsp. *yanninicum* cvs. Larisa and Trikkala); *T. michelianum*; *T. isthmocarpum*; and to different *Medicago* species including alfalfa (*M. sativa*), *M. polymorpha*, *M. truncatula*, *M. litoralis*, and *M. tonata*.

This protocol allows for highly reproducible, robust, and genotype-independent genetic transformation of *Trifolium* species (Fig. 1). Stable meiotic transmission of transgenes has been demonstrated for selected transgenic clovers carrying single T-DNA inserts recovered from *Agrobacterium*-mediated transformation. The average regeneration frequencies of 13 clover cultivars representing three species (*T. subterraneum*, *T. repens*, and *T. pratense*) vary between 65 and 97% (10). Transformation efficiency, calculated as number of

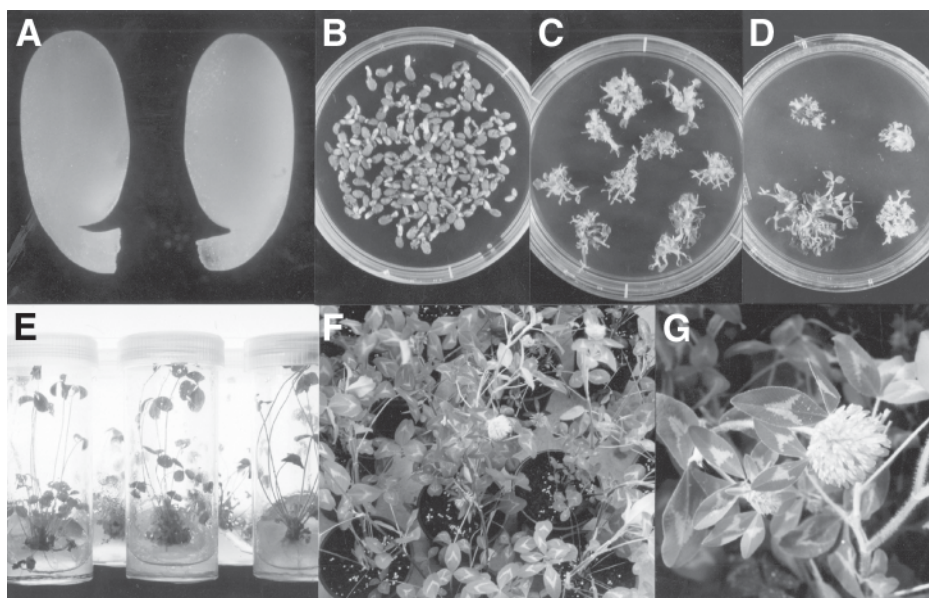


Fig. 1. Transgenic red clover (*Trifolium pratense*) plants obtained from *Agrobacterium*-mediated transformation of cotyledonary explants with a chimeric *nptII* gene. **(A)** Red clover embryo isolated from mature imbibed seed. **(B)** Cotyledonary explants on RM73 medium after 3 d of cocultivation with *A. tumefaciens*. **(C)** Kanamycin-resistant shoots obtained from *A. tumefaciens*-mediated transformation of red clover cotyledonary explants followed by selection on RM73 medium supplemented with 50 mg/L of kanamycin for 2 wk. **(D)** Proliferating kanamycin-resistant shoots regenerated by direct organogenesis in wounded region of cotyledonary explants after 4 wk of antibiotic selection. **(E)** Rooted kanamycin-resistant red clover plantlets growing on RM73 medium 6 wk after transformation. **(F)** Transgenic red clover plants regenerated from cotyledonary explants growing in soil under greenhouse conditions. **(G)** Fertile transgenic red clover plant.

independent transgenic plants confirmed by Southern hybridization analysis per 100 transformed cotyledons, varies between 0.3 and 6%. A further application of this methodology is in “isogenic transformation,” as exemplified for white clover, which provides a control plant with a genetic background identical to that of the transgenic plant.

2. Materials

2.1. *Agrobacterium tumefaciens* Strains and Selectable Marker Genes

Agrobacterium tumefaciens strain AGL1 carrying binary vectors with chimeric neomycin phosphotransferase II (*nptII*), gentamicin acetyltransferase 3

(*aacC1*), or hygromycin phosphotransferase (*hph*) genes as selectable marker genes was used. The chimeric *nptII* gene was under control of either the *nos* or the CaMV 35S promoter. The chimeric *aacC1* and *hph* genes were under control of the CaMV 35S and CaMV 19S promoters, respectively. Binary vectors were derived from pBin19, pKYLX71, or pPZP200 (21,22).

2.2. Culture Media for *Agrobacterium tumefaciens*

1. Luria–Bertani medium: 10 g/L Bacto-tryptone (Becton-Dickinson, Sparks, MD), 5 g/L Bacto yeast extract (Becton-Dickinson), 10 g/L NaCl (Merck). Autoclave, and cool to 45°C before adding the appropriate selective agents.
2. MGL medium: 5 g/L Bacto-tryptone, 2.5 g/L Bacto yeast extract, 1.15 g/L L-glutamic acid, K-salt (Sigma), 5 g/L mannitol (Sigma), 250 mg/L KH_2PO_4 (Merck), 100 mg/L NaCl (Merck), 100 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Sigma).
3. Conservation media: 2.46 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (Sigma), 10 mL/L of 1 M Tris-HCl (Sigma), pH 8.0, 300 mL/L glycerol (Merck).

2.3. Tissue Culture

1. Clover seeds (see **Note 1**).
2. Surface-sterilizing agents: 70% (v/v) ethanol and 25% (v/v) commercial “Domestos” bleach solution (12.5 g/L active chlorine) with 0.1% (v/v) Tween-20.
3. RM73 + acetosyringone (cocultivation media) plates: 4.4 g/L Murashige and Skoog basal medium (Sigma), 5 mL/L of 1 mM thidiazuron stock, 0.5 mL/L of 1 mM naphthalene acetic acid (NAA) stock, 30 g/L sucrose (Merck). Adjust pH to 5.75 with 1 M NaOH and make to volume before adding 8 g/L Bacto-agar. Autoclave, and then cool to 45°C before adding 1 mL/L of a 40 mg/mL acetosyringone stock and pouring into 90 × 90 × 15-mm Petri dishes.
4. RM73 + selection (regeneration media with selection) plates: same as for RM73 plates except that appropriate antibiotics and 1 mL/L of a 250 mg/mL cefotaxime stock are added instead of acetosyringone. Pour into 90 × 90 × 20-mm Petri dishes. Antibiotics: 1 mL/L of kanamycin (50 mg/mL), 1 mL/L of gentamicin (75 mg/mL), or 1 mL/L of hygromycin (50 mg/mL).
5. RIM73 + selection (root-inducing media with selection): 4.4 g/L Murashige and Skoog basal medium, 1.2 mL/L of 1 mM indole-3-butyric acid stock, 15 g/L sucrose. Adjust pH to 5.75 and make to volume, adding 8 g/L Bacto-agar (Becton-Dickinson). Autoclave, and then cool to 45°C before adding 1 mL/L of 250 mg/mL cefotaxime and appropriate antibiotics (as for RM73 + selection plates) and pouring the media into sterile 120-mL tissue culture vessels.
6. RM73 (regeneration media without selection) plates: RM73 without antibiotics except for 250 mg/L cefotaxime.
7. RIM73 (root-inducing media without selection): RIM73 without antibiotics except for 250 mg/L cefotaxime.
8. Acetosyringone (AS): 40 mg/mL stock. For 10 mL stock in dimethylsulfoxide (Sigma): 400 mg 3',5'-dimethoxy-4'-hydroxyacetophenone (Sigma). Filter-steril-

- ize (0.2 μm) and store 1-mL aliquots in 1.5-mL microcentrifuge tubes at -20°C . Protect from light.
9. Indole-3-butyric acid (IBA): 1 mM stock. Dissolve 102 mg powder (Sigma) in 0.5 mL 1 M KOH (Merck). Add purified water to 500 mL. Filter-sterilize and store at 4°C .
 10. NAA: 1 mM stock. Dissolve 96 mg powder (Sigma) in 0.5 mL 1 M KOH. Add purified water to 500 mL. Filter-sterilize and store at 4°C .
 11. Thidiazuron (TDZ): 1 mM stock. Dissolve 110 mg powder (Sigma) in 0.5 mL 1 M KOH. Add purified water to 500 mL. Filter-sterilize and store at 4°C .
 12. 1-mL Cryogenic tubes.
 13. Sterile $90 \times 90 \times 14$ -mm and $90 \times 90 \times 20$ -mm Petri dishes.
 14. Sterile 120-mL tissue culture vessels (cat. no. 75.9922.410, Sarstedt).
 15. Sterile 96-well microtiter plates for cocultivation in the "isogenic transformation" protocol.
 16. Sterile distilled water.
 17. 280 μm Mesh sieve (Saulas, France).
 18. 10-mL Syringes and 18- to 21-G needles.
 19. Dissecting microscopes (Leica Microsystems, Wetzlar, Germany).
 20. Sterile metal spatulas with spoon on one end.
 21. Parafilm.
 22. Transfer to soil: 10.16- or 15.24-cm plastic pots (4–6-inch pots), general-purpose potting mix containing Osmocote 5- to 6-mo (144 g/30 L potting mix) and Nutricote Microfine 70-d (135 g/30 L potting mix) slow-release fertilizers.
 23. Misting bench in the greenhouse for recovery of plants from tissue culture.

2.4. Antibiotic Stocks

1. Carbenicillin: 100 mg/mL stock. For 10 mL stock: 1 g of carbenicillin (Sigma). Filter-sterilize (0.2 μm) and store 1-mL aliquots in sterile 1.5-mL microcentrifuge tubes at -20°C . Working concentration: 100 mg/L.
2. Cefotaxime: 250 mg/mL stock. For 4 mL stock: 1 g of cefotaxime sodium (Sandoz, Vienna, Austria). Filter-sterilize (0.2 μm) and store 1-mL aliquots in sterile 1.5-mL microcentrifuge tubes at -20°C . Working concentration: 250 mg/L.
3. Gentamicin: 75 mg/mL stock. For 10 mL stock: 1090 mg gentamicin sulfate (Sigma). Filter-sterilize (0.2 μm) and store 1-mL aliquots in 1.5-mL microcentrifuge tubes at -20°C . Protect from light. Working concentration: 75 mg/L.
4. Hygromycin: 50 mg/mL stock. For 10 mL stock: 500 mg hygromycin (Sigma). Filter-sterilize (0.2 μm) and store 1-mL aliquots in sterile 1.5-mL microcentrifuge tubes at -20°C . Working concentration: 50 mg/L.
5. Kanamycin: 50 mg/mL stock. For 10 mL stock: 500 mg kanamycin monosulfate (Sigma). Filter-sterilize (0.2 μm) and store 1-mL aliquots in 1.5-mL microcentrifuge tubes at -20°C . Protect from light. Working concentration: 50 mg/L.
6. Rifampicin: 20 mg/mL stock. For 5 mL stock in methanol (Merck): 100 mg rifampicin (Sigma). Filter-sterilize (0.2 μm) and store 1-mL aliquots in sterile 1.5-mL microcentrifuge tubes at -20°C . Working concentration: 20 mg/L.

7. Spectinomycin: 50 mg/mL stock. For 10 mL stock: 500 mg spectinomycin (Sigma). Filter-sterilize (0.2 μ m) and store 1-mL aliquots in sterile 1.5-mL microcentrifuge tubes at -20°C . Working concentration: 50 mg/L.

3. Methods

3.1. Preparation of *Agrobacterium* Cultures

1. Streak *Agrobacterium* AGL1 strain from a glycerol stock onto LB + 20 mg/L rifampicin + 100 mg/L carbenicillin as well as the appropriate antibiotic for selection of bacteria containing the binary vector (e.g., 50 mg/L spectinomycin for pPZP200-derived vectors). Incubate at 28°C in the dark for 2 to 3 d.
2. Transfer three to four individual colonies from the plate into 50 mL cultures of LB + antibiotics used in **step 1**. Incubate at 28°C and 250 rpm in the dark until the culture has reached an OD_{600} of 0.7 (approx 24 h).
3. Dilute an aliquot of the culture 1:1 with conservation medium. Mix well. Transfer 1-mL aliquots into cryogenic tubes. Snap-freeze in liquid nitrogen and store at -80°C . Confirm that the *Agrobacterium* cultures contain the transgene(s) of interest by polymerase chain reaction (PCR).
4. Day 1: add 20 μ L of a 40 mg/mL acetosyringone stock and the appropriate antibiotic for selection of bacteria (e.g., 50 mg/L spectinomycin for pPZP200-derived vectors) to 20 mL of LB and inoculate with 1 mL of thawed *Agrobacterium* conservation stock in a sterile 50-mL centrifuge tube. Cover the flask with aluminum foil to exclude light. Incubate at 28°C and 250 rpm for 24 h.

3.2. Seed Sterilization and Imbibition

1. Day 1: place 1 teaspoon of seeds (approximately 500) into a 280 μ m-mesh sieve and wash seeds for 5 min under running tap water.
2. In a laminar flow hood, transfer seeds to a sterile 120-mL tissue culture vessel with a sterile spatula. Add a magnetic stirrer bar (wiped with 70% ethanol) and add approximately 30 mL of 70% ethanol to the vessel. Stir gently for 5 min. Discard the ethanol appropriately.
3. Add 50 mL of the bleach/Tween-20 solution to the vessel (see **Note 2**).
4. Stir for 20 to 30 min, depending on the cultivar (see **Note 3**).
5. Discard sodium hypochlorite and rinse the seeds six to eight times with approximately 100 mL of sterile distilled water.
6. Add approximately 30 mL of water and cover the vessel with aluminum foil. Incubate the seeds overnight at 15 to 18°C (see **Note 4**).

3.3. Preparation of *Agrobacterium* Inoculum

1. Day 2: centrifuge *Agrobacterium* cultures in a benchtop centrifuge (e.g., Beckman Orbital 500) for 10 min at 1650g. Discard supernatant.
2. Add 20 mL MGL medium plus 50 μ L acetosyringone to each tube. Resuspend by vortexing.

3. Assess bacterial growth, and adjust the OD₆₀₀ to 0.4 to 0.5 with MGL medium plus acetosyringone (see **Note 5**).
4. Incubate at 28°C with shaking (250 rpm) for 2 to 4 h. When the OD₆₀₀ value is 0.7 to 0.8, the *Agrobacterium* suspension is ready to use for transformation.

3.4. Dissection of White Clover Seeds

1. Day 2: place imbibed seeds in a sterile Petri dish under the dissecting microscope. Remove the seed coat and endosperm layer with sterile needles.
2. Cut the hypocotyl, making sure to leave approximately 1.5 mm of the cotyledonary petioles attached to the cotyledons.
3. Separate the cotyledons and transfer them to the Petri dish with 20 mL MGL medium. Continue steps for all cotyledons (see **Notes 6 and 7**).
4. For “isogenic transformation,” see **Note 8**.

3.5. Inoculation of Explants and Cocultivation

1. Day 2: remove the MGL medium from the Petri dish with cotyledonary explants and add the *Agrobacterium* suspension.
2. Seal plates and cover them with aluminum foil.
3. Incubate plates for 45 min at room temperature with gentle shaking.
4. Remove the *Agrobacterium* suspension. Blot cotyledonary explants on one to two pieces of sterilized filter paper and transfer cotyledonary explants onto plates of RM73 media + acetosyringone with sterile forceps, without letting them dry out (see **Note 9** for “isogenic transformation”).
5. Using forceps, distribute the cotyledonary explants so they are not touching each other (no more than 100 explants per dish). Incubate in a growth room at 25°C for 3 d (16-h photoperiod, under fluorescent lights).

3.6. Selection and Regeneration of Transgenic Plants

1. Day 5: after cocultivation, transfer cotyledonary explants from the RM73 + acetosyringone plates with a sterile spoon into 9 × 2.0-cm Petri dishes containing approximately 20 to 30 mL of sterile, distilled water.
2. Wash the cotyledonary explants by gently shaking them in the water-filled Petri dish. Repeat two to three times. Blot cotyledonary explants on sterile filter paper. Do not allow to dry.
3. Transfer the cotyledonary explants individually to 9 × 2.0-cm Petri dishes containing appropriate RM73 selective medium with sterile forceps by inserting the cotyledonary petiole end into the medium (50 explants per plate). Seal the dishes. Incubate at 25°C with a 16-h photoperiod. (see **Note 10** for “isogenic transformation”).
4. Subculture cotyledonary explants every 2 wk for a period of 6 wk by transferring them individually onto fresh dishes containing RM73 selective medium, sealing the dishes with Parafilm, and labeling them appropriately (see **Note 11** for “isogenic transformation”).

3.7. Root Induction

1. Week 7: after 6 wk of selection, excise developing transgenic shoots (with a green base) using a sterile scalpel and transfer to sterile 120-mL tissue culture vessels containing rooting media (RIM73) supplemented with appropriate antibiotics (one plant per vessel). Roots will develop within 8 to 20 d for white clover (see **Notes 12** and **13**).

3.8. Transfer to Soil and Greenhouse Care

1. Weeks 10 to 12: gently remove plantlets with well-formed root systems from culture vessels and wash medium from the roots with tap water.
2. Transfer each plantlet to a 10.16-cm pot containing moist potting mix. Place in a misting bench for 2 wk and then grow under standard greenhouse conditions (21°C, 400 $\mu\text{E}/\text{m}^2/\text{s}$ light condition, 16-h photoperiod).
3. Vegetative growth of plants is supported by adding slow-release fertilizer once per 3 mo and high NPK liquid fertilizer once per fortnight.
4. Upon flowering, plants can be crossed either manually or polycrossed by a controlled bee release leading to seed set typically at rates similar to those of nontransgenic control plants, about 4 to 6 mo post transplantation.

4. Notes

1. This protocol has been used for successful transformation of different *Trifolium* species, including white clover (*Trifolium repens*, cvs. Haifa, Huia, Irrigation, and Mink); red clover (*T. pratense* cvs. Astred, Colenso, Cherokee, Quinequeli, Redquin, and Renegade); subterranean clover (*T. subterraneum* subsp. *brachycalycinum* cv. Clare, subsp. *bubterraneum* cvs. Denmark and Woogenellup, and subsp. *yanninicum* cvs. Larisa and Trikkala); *T. michelianum*; and *T. isthmocarpum*.
2. Adhere to good laboratory practices required for handling transgenic organisms. Wear gloves and eye protection, since sodium hypochlorite is corrosive.
3. The duration of this step is within the 20 to 30 min range but may require evaluation for different cultivars or seed batches. The age of the seed may affect imbibition time. Seed that has been harvested or stored under suboptimal conditions may have internal microbial contamination that surface sterilization will not control effectively.
4. Imbibition at 4°C for 18 or more hours is optimal for *T. repens* cv. Mink.
5. To assess bacterial growth, aliquot 500 μL of suspension derived from each pellet into a cuvet. Also aliquot 500 μL of MGL medium into a cuvet for a blank. Be ensure to record the identity of the culture on each cuvet.
6. Clean a laminar flow hood thoroughly with 70% v/v ethanol. Place in the hood: a microscope, a vessel containing ethanol, and a teaspoon. Close doors and UV-irradiate for 10 min. Turn off UV light, open doors, and switch on fan for 20 min before starting work. All subsequent steps are to be done in the laminar flow hood.

7. If seeds become dry during dissection, add a small quantity (1–2 mL) of autoclaved water. It is important not to let the seeds dry out.
8. The ‘isogenic transformation’ method is based on separate regeneration of both cotyledonary explants from the same seed (i.e., the same genotype). One of them is inoculated with *Agrobacterium* for transformation, and the second one is used directly for regeneration (without *Agrobacterium*-mediated transformation) as an isogenic control. After dissection of a seed, place one of the cotyledonary explants in a numbered well of a sterile 96-well cell microtiter plate containing MGL medium for transformation. Draw a numbered grid on the base of an RM73 (regeneration, no antibiotics except cefotaxime) plate, and place the other cotyledonary explant from the same seed, which will not be transformed, directly onto a numbered position on this plate. Repeat the process for the remaining seeds, taking care to keep track of pairs of untransformed and transformed cotyledonary explants.
9. For ‘isogenic transformation’, draw a numbered grid on the base of a RM73 + acetosyringone (cocultivation) plate and transfer transformed explants directly from numbered wells of the 96-well microtiter plate to numbered positions of the cocultivation plate with a sterile 18- to 21-G needle on a 10-mL syringe.
10. For “isogenic transformation,” draw numbered grids on the base of a RM73 + selection plate and transfer transformed explants from the RM73 + acetosyringone plate to corresponding positions on the RM73 + selection plate.
11. For “isogenic transformation,” regeneration of untransformed partner cotyledons requires RM73 plates with no antibiotics except for cefotaxime. A numbered grid needs to be drawn on the base of these plates, and explants must be transferred from the corresponding positions of one plate to another. Regeneration of the untransformed partner cotyledon occurs faster than regeneration of the transformed cotyledon, so more transfers are necessary.
12. If shoots are too small for transfer to RIM, they can be dissected out with a sterile scalpel and transferred to another RM73 plate with selection. Draw a circle in pen on the base of Petri dishes around multiple shoots derived from the same explant, which might not be independent transformants.
13. For “isogenic transformation,” transfer untransformed partner cotyledonary explants to RIM73 with no antibiotics except for cefotaxime.

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Peas (*Pisum sativum* L.)

Jan Grant and Pauline Cooper

Summary

In this chapter we describe a robust method for transformation of peas that has been successfully used in our laboratory since 1992. Using immature pea seed collected from field- or greenhouse-grown plants, we have produced transgenic lines for over 30 genotypes including named pea cultivars and advanced breeding lines. This method uses immature cotyledons as the explant, and the transformation efficiency is in the range 0.2 to 13.5% of cotyledons producing at least one independently transformed line. *Agrobacterium tumefaciens* strains AGL1 and KYRT1 are the most successful in our procedure, and kanamycin, phosphinothricin, and hygromycin are reliable selectable markers. Potentially useful genes have been introduced for pest and disease resistance, altering quality traits, and investigating metabolic pathways and are being studied in transgenic pea lines.

Key Words: Peas; *Pisum sativum*; transformation; immature cotyledons; legumes; *Agrobacterium tumefaciens*; transgenic plants.

1. Introduction

Peas (*Pisum sativum* L.) are cultivated as food for human consumption, and as feed for animals and are an important component of arable crop rotations. The peas bred for human consumption—garden or vining peas—differ from those bred for forage and dry pea products, which include maple, marrowfat, blue, and white. As with many legumes, peas are regarded as a crop that is ‘recalcitrant’ to transformation. However, it is now 14 yr since pea transformation was first reported by Puonti-Kaerlas et al. (1). More efficient methods have since been developed, and these form the basis of the protocols that are currently used (reviewed in ref. 2). These methods use *Agrobacterium tumefaciens* and differ particularly in the starting explant type, with immature tissue being the most successful. Schroeder et al. (3) used longitudinal slices of immature embryos, Grant et al. (4) used immature cotyledons, and Bean et al.

(5) used injection of *Agrobacterium* cultures into cotyledonary nodes of germinating seeds. All these methods and their later variations have led to the production of transgenic plants containing genes of agricultural and scientific importance with or without selectable marker genes (6–10). Besides explant type, another critical factor for successful transformation is the *Agrobacterium* strain used. The most successful strains are AGL1, EHA105, and KYRT1 (11).

The method described here uses immature cotyledon explants, and transgenic plants for over 30 cultivars or breeding lines have been produced. Rather than developing a protocol that was optimal for specific genotypes, we have taken a generalist approach, which means that agriculturally important lines being developed by plant breeders can be transformed as needed. This method is robust for different pea genotypes and also for plasmid constructs. We have used at least 25 plasmids derived from a range of vector backbones, and all have produced transgenic plants. In addition, we have found only small operator differences in efficiency of production of transgenic plants. An average operator can cocultivate 600 to 800 explants in a normal working day. Transformation efficiency in our system is defined as the number of polymerase chain reaction (PCR)-positive plantlets per cotyledonary explant, with all positive PCR-plants from a single cotyledon counted as one positive. In our system the transformation efficiency varies from 0.2 to 13.5%. The wide range in efficiency depends on the pea genotype and on the *Agrobacterium* strain.

2. Materials

2.1. Plant Material

Immature pea pods from field or greenhouse. We have successfully used cotyledons from all pea types, process peas, maple, marrowfat, white, blue, snow, and sugar snap peas in this protocol to produce transgenic plants.

2.2. *Agrobacterium* Strains and Vectors

Agrobacterium tumefaciens strain AGL1 containing the desired construct (see **Note 1**). The following protocol uses as the selectable marker neomycin phosphotransferase (*nptII*) coding sequence with either the nopaline synthase (*nos*) or the cauliflower mosaic virus 35S (CaMV35S) promoter.

2.3. Stock Solutions

1. Timentin: 200 mg/mL stock solution. (SmithKline Beecham, Australia). Filter-sterilize and store at -20°C .
2. Kanamycin sulfate: 100 mg/mL stock solution: Filter-sterilize and store at -20°C .
3. Streptomycin: 100 mg/mL stock solution. Filter-sterilize and store at -20°C .
4. 40 and 100 mg/mL solutions acetosyingone in dimethyl sulfoxide (DMSO). Make up in a sterile container and store at -20°C .

2.4. Media

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. Store at room temperature.
2. B5 medium (12): 2.5 g/L KNO₃, 0.134 g/L (NH₄)₂SO₄, 0.15 g/L CaCl₂·2H₂O, 0.17 g/L NaH₂PO₄·2H₂O, 0.25 g/L MgSO₄·7H₂O, 3 mg/L H₃BO₃, 13.2 mg/L MnSO₄·4H₂O, 2.0 mg/L ZnSO₄·7H₂O, 0.75 mg/L KI, 0.25 mg/L Na₂MoO₄·2H₂O, 0.025 mg/L CoCl₂·6H₂O, 0.025 mg/L CuSO₄·5H₂O, 8000 mg/L EDTA (FeNa salt), 100 mg/L myoinositol, 1.0 mg/L nicotinic acid, 1.0 mg/L pyridoxine-HCl, 10.0 mg/L thiamine-HCl.
3. Cocultivation medium: B5 medium plus 1.3 mg/L 6-benzylaminopurine (BA), 30 g/L sucrose, 8 g/L agar (Bacto), pH 5.5 and 20 mg/L acetosyringone (add after autoclaving 200 µL of 100 mg/mL stock in DMSO).
4. Regeneration medium: B5 medium plus 1.3 mg/L BA, 30 g/L sucrose, 8 g/L agar (Bacto), pH 5.8, 200 mg/L timentin, and 75 mg/L kanamycin sulfate (see **Note 1**).
5. Rooting medium: B5 medium plus 30 g/L sucrose, 8 g/L agar (Bacto), pH 5.8, 15 mg/L indole acetic acid, and 200 mg/L timentin.
6. Root selection medium: B5 medium plus 30 g/L sucrose, 8 g/L agar (Bacto), pH 5.8, 50 mg/L kanamycin sulfate, and 200 mg/L timentin.
7. Root elongation medium: B5 medium plus 30 g/L sucrose, 8 g/L agar (Bacto), pH 5.8, and 200 mg/L timentin.
8. MS medium (13): 1900 mg/L KNO₃, 1650 mg/L NH₄NO₃, 440 mg/L CaCl₂·2H₂O, 170 mg/L KH₂PO₄, 370 mg/L MgSO₄·7H₂O, 6.2 mg/L H₃BO₃, 22.3 mg/L MnSO₄·4H₂O, 8.6 mg/L ZnSO₄·7H₂O, 0.83 mg/L KI, 0.25 mg/L Na₂MoO₄·2H₂O, 0.025 mg/L CoCl₂·6H₂O, 0.025 mg/L CuSO₄·5H₂O, 8000 mg/L EDTA (FeNa salt), 100 mg/L myoinositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine-HCl, 0.1 mg/L thiamine-HCl, 2.0 mg/L glycine.
9. Multiplication medium: MS medium plus 1.3 mg/L BA, 30 g/L sucrose, 8 g/L agar (Bacto), pH 5.8, and 200 mg/L timentin.

2.5. Other Supplies

1. 100 mL of sterile distilled water in jars.
2. Glad® Wrap plastic food wrap, each roll cut horizontally into 3-cm sections. Used for sealing Petri plates containing explants.
3. Potting mix: to 60 L each of peat, bark, and pumice or perlite add 400 g dolomite lime, 300 g Osmocote®, 75 g superphosphate, 50 g potassium sulfate. Fill plastic pots (15 cm high × 14.5 cm diameter) $\frac{3}{4}$ full with this mix and then top with pumice or perlite.
4. TPS buffer: 100 mM Tris-HCl, pH 9.5, 1 M KCl, 10 mM EDTA.

3. Methods

3.1. *Agrobacterium* Preparation

1. Grow *Agrobacterium tumefaciens* culture overnight in 50 mL LB/MES broth with 50 μ L streptomycin and 50 μ L of 40 mg/mL acetosyringone. Add appropriate amount of kanamycin for *A. tumefaciens* strain. For AGL1 (14), use 50 μ L kanamycin (see Note 2). Put on shaker at 27°C and 100 rpm.
2. The next morning measure the *A. tumefaciens* culture into centrifuge tubes and spin at 3220g for 6 min. Discard the supernatant, add to fresh LB/MES broth + AS, as above, and adjust to the required density—OD 550 nm = 0.35 to 0.40. Return to shaker (can be at room temperature); the culture is now ready to use.

3.2. Explant Preparation

1. From the field or greenhouse collect pea pods that are green and smooth. The pea seed itself should be at maximum size and at “eating ” stage. The pea cotyledons should show no or very little starch forming at the center of the cotyledon. This is approx 2 to 3 wk after flowering.
2. Sterilize the pods by first rinsing in 70% alcohol and then into 15% commercial bleach (4.8 g/100 mL sodium hypochlorite) with 1 to 2 drops Tween-20 on a shaker for 20 min. Rinse pods three times with sterile distilled water.

3.3. Inoculation, Cocultivation, and Selection

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

1. Pipet 5 to 7 mL of the *Agrobacterium* broth into a sterile 60-mm Petri plate.
2. Cut the pod with along one side with a scalpel and remove the peas to a Petri plate. Collect about 25 peas in a Petri plate (see Note 3).
3. Cut each pea in half and discard the half away from the embryo axis.
4. Remove the seed coat and place the pea cut surface down. Dip the scalpel blade in the *Agrobacterium* broth and cut out the embryo at the embryo attachment sites on either side of the embryonic axis and between the cotyledon and the cotyledonary node (Fig. 1A).
5. Place the cotyledon pieces in the broth in the Petri plate and leave for 1 h.

Fig. 1. (opposite page) Pea transformation process. (A) The place where the pea seed is cut. Two cuts are made at the attachment sites of the embryo axis to the cotyledons. (B) Callus has formed where the embryo axis was cut out. At this stage the remaining cotyledon piece can be cut off from the callus. (C) Shoots developing from the callus. Green healthy shoots can be placed on root initiation medium. (D) Root selection for transgenic shoots. The pot on the left shows elongated roots and shoots of transgenic lines, and the pot on the right shows smaller plantlets with short roots that have stopped growing. (E) Transgenic lines growing in the greenhouse.

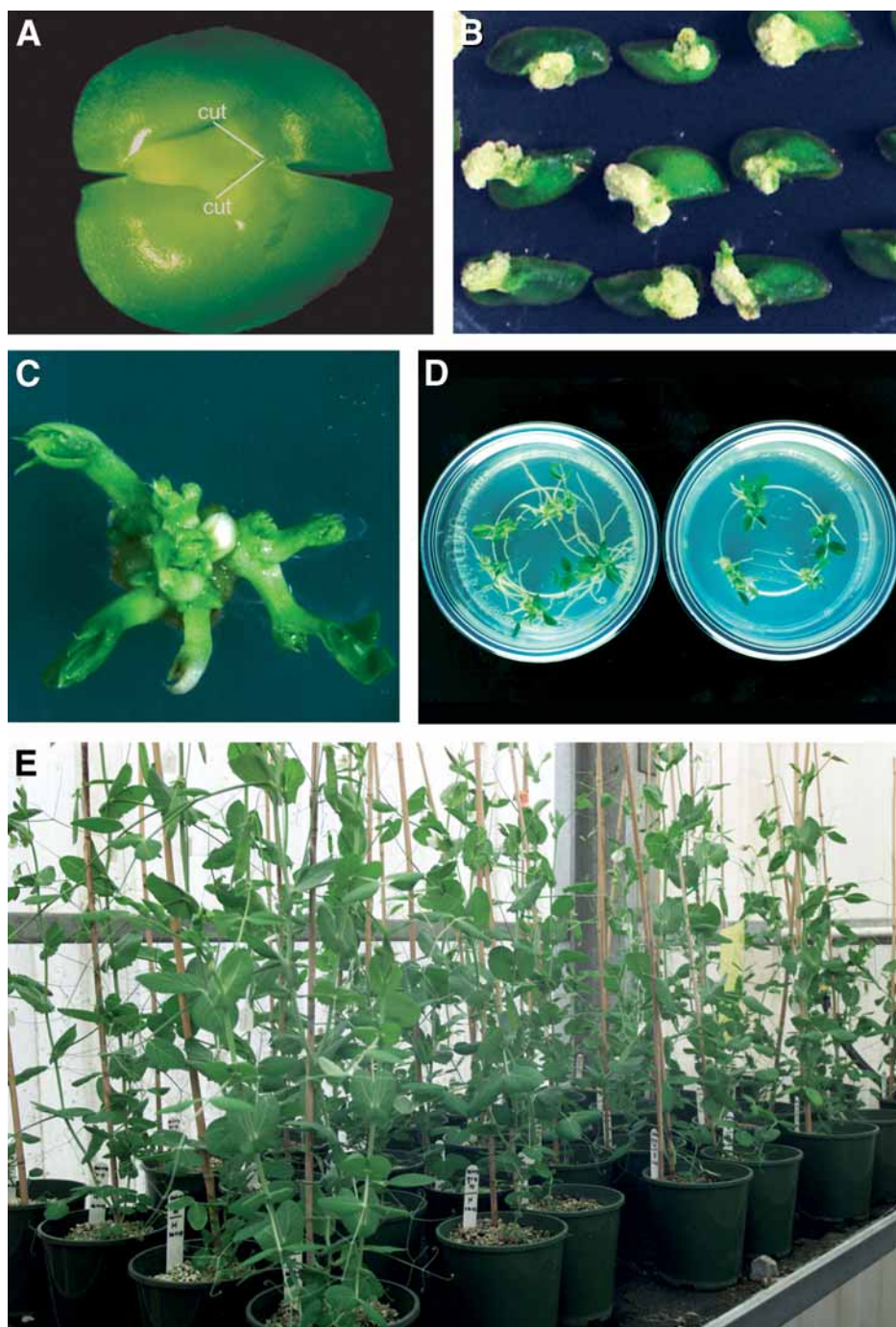


Fig. 1.

6. Remove each cotyledon and place cut surface down on cocultivation medium, usually 50 explants per Petri plate. The growth conditions are the same for all the tissue culture steps. Temperature is constant at 22°C. The lighting regime is a 16-h day with cool white fluorescent light at 30 $\mu\text{mol}/\text{m}^2/\text{s}$ and 8-h night
7. After 4 d put the cotyledons from each Petri plate into a jar of sterile distilled water (one plate per jar). Shake the jar, tip off the water, and add fresh, clean sterile water. Repeat three to four times until the water is clear. Discard and autoclave all rinsing water.
8. Add 400 μL timentin (100 mg/mL) to 100 mL sterile water, add to the cotyledon explants, and let stand for 30 min. Tip off water and timentin, autoclave, and discard.
9. Plate cotyledon explants cut surface down onto regeneration medium (25 explants per plate) and subculture at 2-wk intervals.
10. After 3 to 4 wk, when callus has formed at the site where the embryo axis was removed, cut off the remains of the cotyledon with a scalpel and place the callus onto regeneration medium (**Fig. 1B**).
11. Continue to subculture on regeneration medium at 2-wk intervals.
12. When the developing green shoots reach more than 1 cm, cut off and place on root initiation medium for 5 d (**Fig. 1C**) (see **Note 4**).
13. Place shoots onto root selection medium until roots elongate (**Fig. 1D**). Roots of transformed shoots should elongate approximately 1 cm per day. If roots do not elongate, but shoot looks green, perform PCR to confirm status. If a shoot is pale or brownish, regard as negative (see **Note 5**).
14. Place the growing shoot onto multiplication medium. Cut shoots and divide clumps at 3-wk intervals (see **Note 6**).
15. Root the clonally propagated shoots in the root initiation medium for 5 d and then grow on in root elongation medium before planting in the greenhouse (see **Note 7**).

3.4. Greenhouse Care

1. The greenhouse conditions we use for the plants are 22°C for a minimum 16-h day and 14°C for an 8-h night.
2. To transfer the plantlets from agar into the greenhouse, gently wash the agar off the roots, plant one plantlet per pot, and cover.
3. For covering the plantlets on transfer to soil, we use plastic tubs 95 mm in diameter and 60 mm high.
4. After about a week, when the plants have started growing, one side of the tub is lifted to allow air circulation and gradual reduction of humidity. Two days later the tub is removed completely. Once the plants are established, we do not move the pots so as not to disturb any roots that may have grown out of the bottom of the pot.
5. Water to keep plants moist but not wet. Fertilize two or three times during the growing period with Plantosan® (Aglukon, Düsseldorf, Germany) (**Fig. 1E**).
6. When flowering has finished and pods are filled and drying off, reduce watering so that pods are completely dry for harvesting. Normally we expect 15 to 30 seeds

per plant (range is 0–100), and it usually takes 4 mo from transplanting into the greenhouse to seed harvest. This is also dependent on cultivar and season.

3.5. Confirmation of Transformation

3.5.1. DNA Extraction

1. DNA is extracted for PCR from in vitro pea leaves using a very rapid method developed by Thompson and Henry (15) (see **Note 8**).
2. Dispense 20 μ L TPS buffer into 0.6-mL microtubes or PCR plates.
3. Add 2-mm² piece of leaf material and mash two to three times using a sterile forceps or pipet tip.
4. Centrifuge briefly to push material into liquid.
5. Heat to 96°C for 10 min in a PCR machine.
6. Centrifuge for 1 min at 16,000g, remove supernatant to new tube, and use as template in PCR. Can be stored at 4 to 8°C for 2 to 3 wk.

3.5.2. PCR Reaction

1. Prepare a 25 μ L PCR reaction mix as follows (see **Note 9**):
 - a. 1.5 μ L of 10X PCR buffer (NEB cat. no. M0267L).
 - b. 3.0 μ L of 25 mM MgCl₂.
 - c. 4 μ L of dNTPs mixture (2 mM).
 - d. 0.125 μ L of primers (20 μ M) (see **Note 10**).
 - e. 0.3 μ L Taq polymerase (5U/ μ L).
 - f. 14.45 μ L H₂O.
 - g. To each reaction, add 1.5 μ L template DNA.
2. PCR conditions for *nptII* gene are:
 - a. 94°C for 3 min.
 - b. 94°C for 30 s.
 - c. 60°C for 30 s.
 - d. 72°C for 45 s.
 - e. Do **steps b to d** for 30 cycles.
 - f. 72°C for 7 min.
3. For *virG* PCR, change the annealing temperature (**step c**) to 45°C.
4. Run 12 μ L of product on 1% agarose gel.

4. Notes

1. We have also successfully used phosthriothricin at 4 to 8 mg/L, and hygromycin at 2 mg/L to select transgenic peas. We have used many different constructs and vectors. They include derivatives of pGA643, pBin 19, pBinAR, pBin PLUS, pCAMBIA, pGPTV, and pJH212-T.
2. Different *Agrobacterium* strains that are successful include KYRT1 (16). This strain is not completely disarmed but does give a significant increase in the transformation efficiency. Other workers have success with EHA105.

3. It is preferable that the cotyledons do not dry out while sitting in the Petri plate, so the number depends on how quick the operator is.
4. From one cotyledon, more than one independent transgenic shoot can be obtained. However, these can only be distinguished by Southern analysis. For efficiency, we keep one PCR-positive line per cotyledon. The original cotyledon can then be discarded earlier. If material is limited, many shoots can be tested for independent events using Southern analysis.
5. It can be very difficult to differentiate positive shoots that have not rooted well, so the rapid PCR method is used at this stage to retrieve positive shoots that have not rooted or not rooted well. In addition, possible *Agrobacterium* contamination can also be detected at this stage by rapid PCR using the *virG* primers.
6. Primary transgenic shoots can be maintained for several years in the growth rooms as long as they are regularly subcultured at 3- to 5-wk intervals depending on the genotype.
7. Rooting shoots from the cultures that are maintained on multiplication medium means that the number of shoots planted can be related to the number of seeds required. Furthermore, shoot material maintained on multiplication medium can be tested for activity of the gene of interest instead of waiting for the T₁ generation. This depends on the action of the introduced gene.
8. Other material used successfully as the PCR template includes immature embryos and dried cotyledons and roots.
9. Buffer concentration is reduced and MgCl₂ increased owing to use of TPS extraction buffer. Conditions may have to be optimized for different species.
10. Primers for *nptII* are y: 5'-GAGGCTATTCGGCTATGACT-3', and f: 5'-CCC CTGATGCTCTTCGTCCA-3' and for *virG* primers they are 5'-GCG GTA GCC GAC AG-3' and 5'-GCG TCA AAG AAA TA-3'.

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Peanut (*Arachis hypogaea* L.)

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Summary

Arachis hypogaea (peanut, groundnut), an annual oil seed belonging to the Leguminosae family and the Papilionacea subfamily, is a legume native to South America but now grown in diverse environments in six continents between latitudes 40°N and 40°S. *Arachis hypogaea* can grow in a wide range of climatic conditions. The low yields of this crop are mainly attributed to unreliable rainfall patterns with frequent droughts, lack of high-yielding adapted cultivars, damage by diseases and pests, poor agronomic practices, and limited use of inputs. Genetic engineering approaches have been shown to be comparatively fast, leading to better isolation and cloning of desired traits for combating the various biotic and abiotic stresses. This chapter describes an *Agrobacterium*-mediated transformation protocol in peanut using the cotyledon system. The system described here is potentially applicable to a vast range of genotypes with a high transformation frequency of >70% based on the preliminary molecular data, indicating the production of a large number of independently transformed transgenic plants. The method reported here provides opportunities for crop improvement of this important legume crop via genetic transformation.

Key Words: *Agrobacterium tumefaciens*; *Arachis hypogaea*; genetic transformation; groundnut; Indian peanut clump virus; legume; peanut; shoot regeneration; transgenic plants.

1. Introduction

Legumes are important sources of dietary protein and fats in developing countries of the semiarid tropics, where peanut, or groundnut (*Arachis hypogaea* L.), is one of the important food legume crops (1). There are several constraints to the productivity of the peanut crop that result in great economic losses annually (2). Conventional plant breeding techniques and methodologies have not been successful in imparting resistance against various biotic and abiotic stresses owing to species barriers in the natural system. However,

genetic engineering approaches have been shown to be comparatively fast, leading to better isolation and cloning of desired traits for combating biotic and abiotic stresses. The genetic transformation approach allows for the introduction of novel genes for disease and pest resistance, viral resistance, abiotic stress tolerance, and nutritional improvement that are not normally accessible by conventional breeding, i.e., are limited by sexual incompatibility (3). Effective regeneration and transformation systems are the prerequisites for a successful genetic transformation. Stable engineered resistance requires the production of numerous independent transformants to allow the selection of those with the appropriate level of gene expression (2). There are numerous reports of tissue culture and regeneration of peanut from various explants (5–9). Regeneration via somatic embryogenesis has also been reported as one of the promising methods for transformation studies in peanut (10–13). A direct regeneration system in peanut has an advantage, owing to the rapidity of morphogenesis and no requirement for frequent subculture; in addition, *de novo* production of shoot primordia is extremely rapid and initially synchronous with the period of cellular differentiation (3). Such a regeneration system favors easy accessibility of *Agrobacterium* to the meristematic cells, which are mainly surface cells during the initial cocultivation for genetic transformation.

Transformation of plants involves the stable introduction of DNA sequences, usually into the nuclear genome of cells capable of giving rise to a whole transformed plant. Transformation efficiencies are frequently directly related to the tissue culture response, and therefore highly regenerative cultures are often transformation competent.

Peanut transformation, like all other transformation systems, relies on some common key elements. The major components for the development of transgenic plants are: (1) the development of reliable tissue culture regeneration systems, (2) preparation of gene constructs and transformation with suitable vectors, (3) efficient techniques of transformation for the introduction of genes into the crop plants, (4) recovery and multiplication of transgenic plants, (5) molecular and genetic characterization of transgenic plants for stable and efficient gene expression, (6) transfer of genes to elite cultivars by conventional breeding methods if required, and (7) evaluation of transgenic plants for their effectiveness in alleviating biotic and abiotic stresses without being an environmental biohazard (14).

Here we describe an efficient transformation system of peanut with high transformation frequency based on an earlier published procedure (4) using cotyledon explants forming adventitious shoot buds in different peanut genotypes. This transformation protocol has been optimized for the development of a transgenic peanut capable of producing fertile plants by *Agrobacterium tumefaciens*-mediated transformation that is potentially applicable to a wide

range of peanut genotypes. The protocol uses cotyledon explants from pre-soaked mature seeds that can produce multiple adventitious buds that are highly amenable to *A. tumefaciens* infection and result in large numbers of transgenic events from a given experiment. The system results in the production of a large percentage (55% of the putative transgenic plants are positive for gene integration) of transgenic plants.

2. Materials

2.1. Plant Tissue Culture

1. Healthy peanut seeds of variety JL24 obtained from the gene bank of the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT).
2. 70% Ethanol.
3. 0.1% (w/v) Aqueous mercuric chloride.
4. MS medium (15): MS inorganic salts and MS organic constituents:
 - a. Major salts (50X): prepare stock solutions of the major salts of MS medium.
 - i. NH_4NO_3 : dissolve 33 g of NH_4NO_3 in 200 mL of sterile distilled water. Store the stock at room temperature for no more than 1 mo. Use 10 mL of the stock solution for preparing 1 L of the medium.
 - ii. KNO_3 : dissolve 38 g of KNO_3 in 400 mL of sterile distilled water. Store the stock at room temperature for no more than 1 mo. Use 20 mL of the stock solution for preparing 1 L of the medium.
 - iii. KH_2PO_4 : dissolve 3.40 g of KH_2PO_4 in 200 mL of sterile distilled water. Store the stock at room temperature for no more than 1 mo. Use 10 mL of the stock solution for preparing 1 L of the medium.
 - iv. CaCl_2 : dissolve 8.80 g of CaCl_2 in 200 mL of sterile distilled water. Store the stock at room temperature for no more than 1 mo. Use 10 mL of the stock solution for preparing 1 L of the medium.
 - v. $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$: dissolve 7.40 g of $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ in 200 mL of sterile distilled water. Store the stock at room temperature for no more than 1 mo. Use 10 mL of the stock solution for preparing 1 L of the medium.
 - b. Minor salts (100X): weigh the required quantities of the minor salts (83 mg KI, 2230 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 860 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 25 mg $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 2.5 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 2.5 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$) and dissolve in 100 mL of sterile distilled water. Store the stock at 4°C for no more than 1 mo. Use 5 mL of the stock solution for preparing 1 L of the medium.
 - c. Iron (100X)
 - i. $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$: dissolve 3.73 g of the chemical in 1000 mL of sterile distilled water. Store the stock at 4°C for no more than 1 mo. Use 5 mL of the stock solution for preparing 1 L of the medium.
 - ii. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$: dissolve 2.78 g of the chemical in 1000 mL of sterile distilled water. Store the stock at 4°C for no more than 1 mo. Use 5 mL of the stock solution for preparing 1 L of the medium.

OR

- iii. FeNa₂EDTA: dissolve 2 g of the chemical in 500 mL of sterile distilled water. Store the stock at 4°C for no more than 1 mo. Use 5 mL of the stock solution for preparing 1 L of the medium
5. B5 organic constituents (16): dissolve 50 mg nicotinic acid, 50 mg pyridoxine monohydrochloride, and 50 mg thiamine hydrochloride in 250 mL of sterile distilled water. Use 10 mL of this stock for preparation of 1 L medium.
6. Modified MS medium (MMS): MS inorganic salts, B5 organic constituents, 3% sucrose, and 0.8% Difco Bacto agar. The pH of the medium is adjusted to 5.8 prior to autoclaving.
7. Benzyl adenine (BA; Sigma): 1 mM stock. Dissolve 22.5 mg of BA in 1 mL of 1 N NaOH and then bring the volume to 100 mL with autoclaved water. Stock solutions are stored at 4°C.
8. Kinetin (Sigma): 1 mM stock. Dissolve 21.5 mg of kinetin in 1 mL of 1 N NaOH and then make up the volume to 100 mL with autoclaved water. Stock solutions are stored at 4°C.
9. β-naphthalene acetic acid (NAA, Sigma): 1 mM stock. Dissolve 18.6 mg of NAA in a few drops of ethanol and then bring the volume to 100 mL using autoclaved distilled water. Stock solutions are stored in single aliquots at 4°C.
10. 2,4-Dichlorophenoxyacetic acid (2,4-D, Sigma): 1 mM stock. Dissolve 22.1 mg of 2,4-D in a few drops of ethanol, bring the volume to 100 mL with autoclaved distilled water, and store in single aliquots at 4°C.
11. Cefotaxime: 125 mg/mL stock. Dissolve the chemical in distilled water and filter-sterilize the stock prior to use.
12. Kanamycin monosulfate (Sigma): 125 mg/mL stock. Dissolve the kanamycin powder in water, filter-sterilize, and store in aliquots at -20°C for no more than 15 d.
13. Shoot induction medium (SIM): MMS plus 20 μM BA and 10 μM 2,4-D. Pour the medium into 90 × 16-mm sterile disposable plastic Petri plates. Store in a cool place for no more than 15 to 20 d.
14. Shoot elongation medium 1 (SEM): MMS plus 2 μM BA. Pour the medium into 150 × 25-mm-long test tubes plugged with nonabsorbent cotton plugs wrapped in one layer of cheesecloth. Store in a cool place for no more than 15 to 20 d.
15. Root induction medium (RIM): MMS plus 5 μM NAA. Pour the medium in 150 × 25-mm-long test tubes plugged with nonabsorbent cotton plugs wrapped in one layer of cheesecloth. Store in a cool place for no more than 15 to 20 d.

2.2. Bacterial Culture

1. *Agrobacterium* strain and vector: disarmed *A. tumefaciens* strain C58, harboring the plasmid pROKII:IPCVcp containing the coat protein gene and pBI121 containing *uidA* (GUS) as the reporter gene linked to the CaMV 35S promoter, with both the plasmids having the *nptII* gene as a selectable marker under the control of the NOS promoter and polyA sequences within the T-DNA borders.

2. Luria-Bertani medium (LB): 1% Bacto-tryptone, 0.5% Bacto yeast extract, 1% NaCl, 1.5% agar dissolved in deionized water. The pH is adjusted to 7.0 with 5 N NaOH (approx 0.2 mL) prior to autoclaving.
3. Yeast extract medium (YEB): 0.5% Bacto-peptone, 0.1% yeast extract, 0.5% beef extract, 0.5% sucrose, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. The pH is adjusted to 7.0 with NaOH prior to autoclaving.
4. $\frac{1}{2}$ MS containing 3% sucrose. The pH is adjusted to 5.8 before autoclaving.

2.3. Characterization of Putative Transformants

2.3.1. Histochemical Analysis

1. 0.1 M Sodium phosphate buffer: 0.1 M of monobasic sodium phosphate (13.9 g in 1000 mL), 0.1 M of dibasic sodium phosphate (26.85 g in 1000 mL). Add 31 mL of monobasic solution and 69 mL of the dibasic sodium phosphate to a final volume of 100 mL, pH 7.0. Store at 4°C.
2. X-gluc (5-bromo-4-chloro-3-indolyl β -D-glucuronide) solution: dissolve 10 mg of X-gluc in 100 μL of dimethylformamide (DMF) and adjust the volume to 5 mL with 0.1 M sodium phosphate buffer (pH 7.0), 25 μL of 200 mM potassium ferrocyanide, 25 μL of 200 mM potassium ferricyanide, 50 μL of 1 M Na_2EDTA , 100 μL of 0.1% Triton-X, 200 μL of 50 mg/L sodium azide. Store in a dark bottle away from light at 4°C for immediate use.

2.3.2. Genomic DNA Extraction and Purification

1. Extraction buffer: 100 mM Tris-HCl, pH 8.0, 50 mM EDTA, 500 mM NaCl, 10 mM β -mercaptoethanol,
2. 20% Sodium dodecyl sulfate (SDS).
3. 5 M Potassium acetate, pH 5.0.
4. Isopropanol.
5. 10 mg/mL RNase A in 10 mM Tris-HCl, pH 8.0, 15 mM NaCl. Boil for 20 min to inactivate contaminating DNases. Store in aliquots at -20°C .
6. TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.
7. DEAE-cellulose suspension: 7.5% Whatman DE 52, 2 M NaCl, in TE buffer, pH 8.0.
8. Wash buffer: 400 mM NaCl, in TE buffer, pH 7.5.
9. Elution buffer: 2 M NaCl, in TE buffer, pH 7.5.

3. Methods

3.1. *Agrobacterium inoculum* Preparation

1. Disarmed *A. tumefaciens* strain C58, harboring binary plasmids pBI121 and pROKII:IPCV, are maintained on LB agar plates containing 50 mg/L kanamycin and 25 mg/L rifampicin, for use in transformation.
2. Grow single colonies of the strain in 20 mL of YEB supplemented with 50 mg/L kanamycin at 28°C on an orbital shaker (100 rpm) overnight or till the OD reaches 0.6.

3. Centrifuge 10 mL of bacterial cells at 600g for 10 min and resuspend the pellet in 30 mL of $\frac{1}{2}$ strength MS-containing 3% sucrose (1:3 dilution). Store the suspension at 4°C for 1 to 2 h before cocultivation.
4. Shake the bacterial suspension and pour it into sterile Petri plates so as to make a thin film (2–3 mm) at the base of the plate.

3.2. Explant Preparation and Cocultivation

1. Mature seeds of the selected peanut variety are removed from healthy pods and stored at 4 to 10°C prior to use. If the pods are not stored at low temperature, the seeds can lose their viability.
2. Surface-sterilize the seeds by rinsing in 70% ethanol for 1 min followed by treatment with 0.1% (w/v) aqueous mercuric chloride for 10 min. Wash thoroughly four to six times with sterile-distilled water and soak the seeds in sterile water for 4 h before use. Unless noted otherwise, all steps are to be performed in a laminar airflow chamber.
3. Remove the seed coat surgically under aseptic conditions, remove the embryonic axis, and cut each cotyledon into vertical halves (**Fig. 1A**) to obtain the cotyledon explants (see **Note 1**).
4. Immerse the freshly excised cotyledons with their proximal cut ends into the bacterial suspension for a few seconds and implant on SIM with cut ends embedded in the medium for 72 h at $26 \pm 1^\circ\text{C}$ under continuous lighting provided by white cool fluorescent lamps of 60 $\mu\text{E}/\text{m}^2/\text{s}$ light intensity (**Fig. 1B**).

3.3. Maintenance of Cultures and Regeneration of Shoots

1. Transfer the cotyledon explants cocultivated with the *Agrobacterium* solution (five explants per plate) to SIM supplemented with 250 mg/L cefotaxime for 2 wk until multiple shoots appear on at least 70% of the explants. Take the utmost care to embed the cut end of the explant (**Fig. 1C**) into the medium (see **Note 2**).
2. Transfer the explants bearing shoot buds (**Figs. 1D–F**) to SEM containing 250 mg/L cefotaxime and 100 mg/L kanamycin to initiate selection and enrichment of the transformed cells for another 2 wk (see **Notes 3** and **4**). Cultures are maintained at $26 \pm 1^\circ\text{C}$ under continuous light provided by white cool fluorescent lamps with 60 $\mu\text{E}/\text{m}^2/\text{s}$ light intensity.

Fig. 1. (opposite page) Regeneration of adventitious shoots from cotyledon explants of *A. hypogaea* L. **(A)** Embryo axis is removed surgically from the healthy groundnut seeds, and each cotyledon is then cut into vertical halves to obtain the cotyledon explants. **(B)** Cultured explants on shoot induction medium after cocultivation for 48 to 72 h. **(C)** Greening of cotyledons and initiation of shoot bud formation from the explants after 7 to 9 d of culture. **(D)** Induction of adventitious shoot buds from cotyledon explants after 2 wk of culture on SIM showing the swelling of cut ends resulting in shoot buds. **(E)** Development of multiple adventitious shoots from cut end of the cotyledon explants after 3 wk. **(F)** Cotyledon explants bearing the multiple shoot buds after 4 wk of culture.

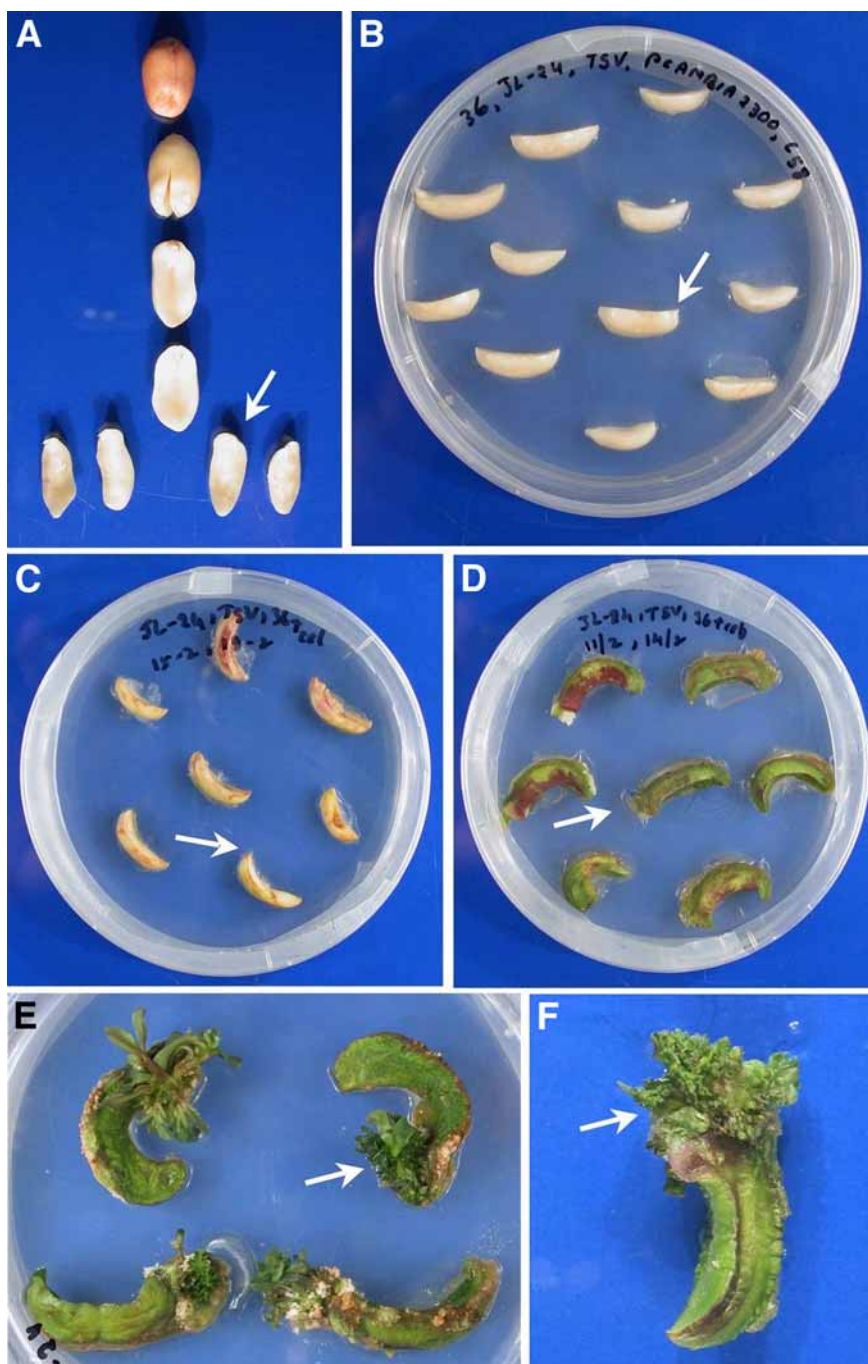


Fig. 1.

3. After 2 wk, excise the proximal parts of the explants containing multiple adventitious shoot buds (**Fig. 2A**) and transfer to SEM containing 125 mg/L kanamycin for two to three subcultures of 4 wk duration each (see **Notes 5** and **6**).
4. Culture the elongated shoots (3–4 cm) on RIM without any antibiotics for rooting of the shoots. It takes about 2–3 wk to obtain multiple adventitious roots on the shoots after culture on RIM (**Fig. 2C**).

3.4. Transplantation and Maintenance of Plants in the Greenhouse

1. Remove the plants gently from the culture tubes and carefully wash the medium using sterile distilled water.
2. Transfer the rooted shoots to pots containing an autoclaved sand and red soil (1:1) mixture covered with plastic bags to maintain high humidity (85%) at 25°C in a growth cabinet with a 16-h photoperiod and 60 $\mu\text{E}/\text{m}^2/\text{s}$ light intensity.
3. Make small holes in the plastic bags after 7 to 8 d for acclimatization of the plants. Remove the plastic bags after 10 d.
4. After 2 wk, transfer the plants to 25-cm-diameter pots containing autoclaved field soil to a contained greenhouse with 26 to 30°C/20 to 25°C day/night temperatures and about a 10- to 12-h photoperiod for flowering and seed set.
5. The plants are irrigated with Hoagland's nutrient solution once a month and routinely irrigated with tap water whenever required.
6. All the transformed plants produced flowers and pods within 120 d and contained viable seeds. Each plant produced up to 40 pods that provided a total of up to 75 seeds per plant when grown in 12-inch-diameter pots. The flowering and maturity may depend on the selected variety.

3.5. Histochemical Localization of uidA Gene Activity in Putative Transformants

1. The primary transformants (T_0) are analyzed for the presence and expression of the introduced genes upon transfer to the greenhouse.
2. Cut free-hand sections of petiole and stem sections of putative transformants growing in vitro or in the greenhouse.
3. Add 200 μL of the X-gluc assay mixture to the tissue sample and infiltrate under vacuum for 3–5 min.
4. Incubate the samples at 37°C for 4 to 24 h in the dark.
5. Stop the reaction by removing the mixture and dehydrating the sample by sequential washing in 70 to 100% ethanol until tissue is devoid of chlorophyll.
6. Examine the tissue for staining under a microscope.

3.6. Molecular Analysis of Putative Transformants

3.6.1. Extraction and Purification of Genomic DNA from Peanut Leaves (4)

1. Collect young leaf tissue (0.5 g) from putatively transformed peanut plants growing in a contained greenhouse. Freeze-dry the samples by immersing in liquid nitrogen and lyophilize for 10 to 15 min.

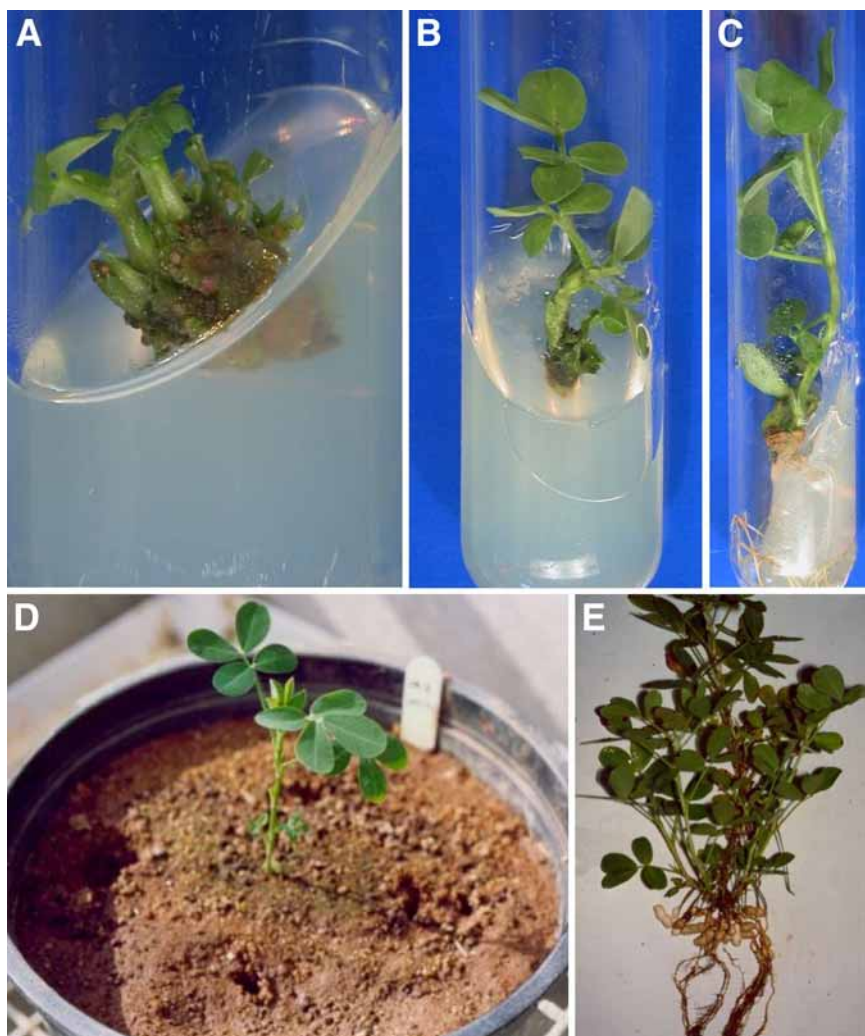


Fig. 2. Development of adventitious shoots into plants. **(A)** Development of multiple adventitious shoots after culture on shoot elongation medium (SEM) after 10 d of culture. **(B)** Elongation of a shoot cultured on SEM after 2 wk. **(C)** Production of multiple adventitious roots on elongated shoots after 2 to 3 wk of culture on root induction medium (RIM). **(D)** A rooted plant transplanted to a pot and maintained under greenhouse conditions after 2 wk of transplantation. **(E)** Mature groundnut transgenic plant with well-developed pods and seeds at the time of harvest.

2. Homogenize, with a mortar and pestle, the dry lyophilized plant material to a fine powder.
3. Transfer the powder with a metallic spatula to a 25-mL polypropylene tube and add 15 mL of extraction buffer and 1 mL 20% SDS. Mix gently and incubate in a water bath for 15 min.
4. Bring the samples to room temperature, add 5 mL of 5 M potassium acetate, mix gently, and incubate on ice for 30 min.
5. Spin the tubes for 20 min at 1800g at room temperature.
6. Transfer the supernatant to another tube, avoiding disturbance to the interphase.
7. Precipitate the DNA with 0.6 vol of isopropanol, mix gently, and incubate for 30 min at -20°C before centrifugation at 1500g for 10 min.
8. Discard the supernatant and wash the pellet in 70% ethanol, followed by air-drying.
9. Add 700 μL TE buffer and 10 μL of RNase (10 mg/mL) and incubate at 37°C for 2 to 3 h.
10. Dilute the DNA with two volumes of distilled water.
11. Add to it 1 mL of DEAE-cellulose suspension with gentle mixing for 3 min so as to maximize the interaction between nucleic acids and the DEAE-cellulose matrix (see **Note 7**).
12. Spin the mixture for 30 s at 500g to allow the sedimentation of DEAE-cellulose particles, to which the nucleic acids have bound.
13. Discard the supernatant carefully and resuspend the pellet in 1.2 mL wash buffer to eliminate the proteins, polysaccharides, and secondary metabolites not bound to DEAE-cellulose.
14. Repeat this step again at least once for better results.
15. Add 0.5 mL of elution buffer to the DEAE-cellulose pellet and mix gently prior to centrifugation at 500g for 30 to 45 s (see **Note 8**).
16. Collect the supernatant in a fresh 1.5-mL microfuge tube and repeat this step once again with 0.3 mL of elution buffer.
17. Carefully pool the supernatants and precipitate using 0.6 vol of isopropanol, followed by centrifugation at 1500g for 10 min at room temperature.
18. Discard the supernatant, wash the pellet with 1 mL of 70% ethanol, and centrifuge at 1800g for 2 min.
19. Discard the supernatant and air-dry the pellet properly. Dissolve the dry pellet in 50 to 100 μL of TE buffer (pH 8.0) for long-term storage at -20°C . Typically, 0.5 g tissue yields approx 15 to 20 μg of purified DNA.

3.6.2. Characterization of Transgenic Plants

1. Carefully number each putative independent transformant arising from a treated explant and maintain separately for subsequent DNA analysis and progression, e.g., generations such as T_1 , T_2 , T_3 , and so on.
2. Ascertain the segregation pattern of the *uidA* gene by using β -glucuronidase (GUS) expression in the T_1 and T_2 generation progenies. PCR analysis of the

introduced genes can also be carried out to confirm the segregation pattern in the progenies.

3. Examine the T-DNA insertion pattern by separately digesting the genomic DNA from each of the putative transformants by using the restriction enzyme that has a single internal site in the plasmid, and resolve on 0.8% agarose gel for transfer onto nylon membranes (HybondN+, Amersham) by a standard protocol.
4. Probe the blots with PCR-amplified fragments of the *nptII* gene labeled with a radioactive or nonradioactive detection system according to the manufacturer's instructions.

4. Notes

1. The embryonic axis should be removed surgically before the explants are prepared.
2. Care should be taken to embed the proximal cut end of the cotyledon into the medium so that it remains in contact with the medium at least for the first 2 wk of culture initiation.
3. Judicious use of selection levels is an important criterion for the recovery of transformed cells, as too high a level would be detrimental even to the transformed cells in early stages.
4. A greater number of shoots can be recovered if the explants are subcultured on SEM1 for one to two extra subcultures.
5. The bleached shoots must be removed with care at every stage of selection to reduce the number of escapes.
6. Use of kanamycin has not been considered very reliable for visual selection at a later stage since some of the nonbleached shoots have been found to be untransformed. However, it does play a selective role in suppression of the shoot bud induction from the untransformed cells.
7. The DEAE-cellulose suspension must be properly mixed with the DNA solution to maximize the interaction between the two.
8. Elution of the DNA should be done at least twice to maximize the yield of the purified DNA.

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Pigeonpea (*Cajanus cajan* L. Millsp.)

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Summary

Pigeonpea [*Cajanus cajan* (L.) Millsp.], also known as redgram, is one of the major grain legume (pulses) crops grown in the semiarid tropics (SAT) extending between 30°N and 30°S; it is the second most important food legume of India. It is cultivated in about 50 countries of Asia, Africa, and the Americas for a variety of uses (food, fodder, fuel wood, rearing lac insects, hedges, wind breaks, soil conservation, green manure, roofing, and so on). The constraints of enhancing its productivity include the damage caused by various fungi, bacteria, viruses, and insect pests. Conventional plant breeding methods have not been successful for the improvement of pigeonpea because of genetic variation and incompatibility among the wild varieties. Genetic engineering technology can therefore be used as an additional tool for the introduction of agronomically useful traits into established varieties. The development of plant transformation techniques has been a major breakthrough in overcoming constraints to achieve precision in genetic manipulation. The development of efficient plant regeneration protocols is a prerequisite for recombinant technology to carry out genetic transformation. This chapter describes an *Agrobacterium*-mediated transformation protocol for pigeonpea, a simple, efficient, and reproducible method that is applicable across diverse genotypes of pigeonpea.

Key Words: *Agrobacterium tumefaciens*; *Cajanus cajan*; legumes; pigeonpea; genetic transformation; redgram; regeneration; transgenic plants.

1. Introduction

Pulses have been indispensable constituents of the Indian diet, and pigeonpea, an important grain legume, stands second in terms of cultivation and yield in India. It is a member of the Fabaceae family, with chromosome number $2n = 22$. It is an important grain legume crop of rain-fed agriculture in the semiarid tropics of the Indian subcontinent and is also widely grown in Southeastern Africa, Latin America, and the Caribbean. Its productivity is constrained

by both biotic (1) and abiotic factors. Conventional plant breeding methods have not been successful in improving pigeonpea because of genetic variation and incompatibility among the wild varieties. The major difference between conventional breeding and biotechnology lies in the speed, precision, reliability, and scope. The development of plant transformation techniques has been a major breakthrough in overcoming constraints to achieve precision in genetic manipulation. With the establishment of vector-mediated and direct gene transfer methods, genes from taxonomically distant and or unrelated donors have been incorporated into plants. In pigeonpea there are numerous reports on tissue culture and regeneration (2–4) and few reports on genetic transformation (5–9).

Reliable tissue culture and plant regeneration systems play an important role in routine production of transgenic plants (10,11). Direct regeneration is preferred to indirect regeneration as the length of the callus phase is negatively correlated with regeneration ability: the somaclonal variation can influence the phenotype of the regenerated shoots. Here we describe a simple and highly reproducible *Agrobacterium*-mediated genetic transformation of pigeonpea using leaves as explants (12). Five- to 6-d-old leaf explants from germinated seedlings are cocultivated with *Agrobacterium tumefaciens* strain C58 harboring the plasmid pCAMBIA1301 carrying the genes encoding for hygromycin phosphotransferase (*hpt*) and β -glucuronidase (*uidA*). The putatively transformed shoots are selected on selection medium with hygromycin as the selection agent. The shoots are rooted on rooting medium and transferred to pots containing a 1:1 sand and soil mixture. Stable expression and integration is confirmed by GUS assay, polymerase chain reaction (PCR), and Southern blot analysis. With this protocol we obtain an average of 75% transformation efficiency, i.e., 75 antibiotic-resistant PCR-positive independent lines from 100 infected plants.

2. Materials

2.1. Tissue Culture

1. Healthy pigeonpea seeds of variety ICPL 88039 obtained from the gene bank of the International Crop Research Institute for the Semi-Arid Tropics (ICRISAT).
2. 70% v/v Ethanol.
3. 0.1% w/v Mercuric chloride, Tween-20.
4. MS medium (13): MS inorganics + MS organics + 3% sucrose + 0.8% Difco Bacto agar. Adjust the pH of the medium to 5.8 with 1 N NaOH prior to autoclaving (Table 1).
5. Kinetin (Sigma): 1 mM stock. Weigh 21.52 mg of kinetin and dissolve in few drops of 1 N HCl, add autoclaved water to 100 mL, filter-sterilize, and store at 4°C in aliquots.

Table 1
Composition of Murashige and Skoog (1962) Basal Medium (MS)^a

Salt	Concentration required (mg/L)	Stock (g/L)	Use (mL/L)
Major salts			
NH ₄ NO ₃	1650	165	10
KNO ₃	1900	95	20
KH ₂ PO ₄	170	17	10
CaCl ₂	440	44	10
MgSO ₄ ·7H ₂ O	370	37	10
Minor salts			
H ₃ BO ₃	6.20	0.620	10
KI	0.83	0.083	10
MnSO ₄ ·H ₂ O	22.3	2.230	10
ZnSO ₄ ·7H ₂ O	8.6	0.860	10
Na ₂ MoO ₄ ·2H ₂ O	0.25	0.025	10
CuSO ₄ ·5H ₂ O	0.025	0.0025	10
CoCl ₂ ·6H ₂ O	0.025	0.0025	10
Iron			
Na ₂ EDTA·2H ₂ O	37.3	3.73	10
FeSO ₄ ·7H ₂ O	27.8	2.78	10
or			
FeNa ₂ EDTA	40	4.00	10
Organics			
Glycine	2.0	0.200	10
Nicotinic acid	0.5	0.050	10
Thiamine HCl	0.1	0.010	10
Pyridoxine HCl	0.5	0.050	10
m-Inositol	100	10.0	10

^aSee [ref. 13](#).

6. N⁶-benzyl adenine (BA; Sigma): 1 mM stock. Dissolve 22.5 mg of BA in few drops of 1 N NaOH, add autoclaved water to 100 mL, filter-sterilize, and store at 4°C in aliquots.
7. Giberellic acid (GA₃; Sigma): 1 mM stock. Dissolve 34.63 mg of GA₃ in 1 mL of sterile water, filter-sterilize, and store at 4°C in aliquots.
8. Indole-3-acetic acid (IAA; Sigma): 1 mM stock. Dissolve 17.52 mg of IAA in a few drops of 1 N NaOH, add autoclaved water to 100 mL, filter-sterilize, and store at 4°C in aliquots.
9. Cefotaxime: 125 mg/mL stock. Dissolve 125 mg of cefotaxime in 1 mL distilled water, and filter-sterilize the stock prior to use.

10. Kanamycin monosulfate (Sigma): 100 mg/mL stock. Weigh 100 mg of kanamycin, dissolve in 1 mL of distilled water, filter-sterilize, and store at -20°C for not more than 15 d.
11. Hygromycin (Sigma): 50 mg/mL stock. Weigh 50 mg of hygromycin, dissolve in 1 mL of distilled water, and store at -20°C .
12. Shoot induction medium (SIM): MS medium + 250 mg/L cefatoxime + $5\text{ }\mu\text{M}$ BA + $5\text{ }\mu\text{M}$ kinetin.
13. Shoot development medium (SDM): MS medium + 250 mg/L cefotaxime + $0.25\text{ }\mu\text{M}$ BA + $0.25\text{ }\mu\text{M}$ kinetin.
14. Shoot elongation medium (SEM): MS medium + 250 mg/L cefotaxime + $0.58\text{ }\mu\text{M}$ GA₃.
15. Root induction medium (RIM): MS basal medium + 1% sucrose.

2.2. *Agrobacterium tumefaciens* Strains and Selectable Markers

We have used C58 as the *Agrobacterium* strain harboring the pCambia1301 plasmid with the *hpt* and *uidA* genes as selectable and screenable markers.

2.3. Culture Media for *Agrobacterium tumefaciens*

1. Yeast extract medium (YEB): for 1 L. Weigh and dissolve 5 g of Bacto-peptone, 1 g of yeast extract, 5 g of beef extract, 5 g of sucrose, and 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ in 800 mL of water. Adjust the pH to 7.0 with 1 N NaOH, make the volume to 1000 mL, and autoclave prior to use. Then cool to 50°C before adding the appropriate selective agent (depending on the vector).
2. LB agar: weigh and dissolve 10 g of Bacto-peptone, 5 g of yeast extract, and 10 g of sodium chloride in 800 mL of water, adjust the pH to 7.0 with 1 N NaOH, add 15 g of agar, make the volume to 1000 mL, and autoclave. Then cool to 50°C before adding the appropriate selection agent (depending on the vector).

2.4. GUS Assay (14)

1. 100 mL of 1 M Sodium phosphate stock solution: dissolve 14.2 g of Na_2HPO_4 (dibasic sodium phosphate) and 13.8 g of $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (monobasic sodium phosphate) in approximately 90 mL of sterile distilled water. Adjust the pH to 7.0 using 1 N NaOH. If necessary to reduce the pH, use phosphoric acid. Bring the volume to 100 mL with distilled water.
2. Assay mixture: 100 mL of X-Gluc solution. Weigh out 0.052 g of X-Gluc. Dissolve the X-Gluc in about 50 mL of sterile distilled water. Add 5 mL of 1 M sodium phosphate stock solution and 0.1 mL of the detergent Triton X-100 and mix. Bring the volume up to 100 mL with distilled water. Store the solution in the dark in the refrigerator (see **Note 1**).

3. Methods

3.1. Explant Preparation and Culture Conditions

1. Sterilize pigeonpea seeds by rinsing in 70% ethanol for 2 min and 0.1% mercuric chloride containing 1 to 2 drops of Tween-20 for 8 min on a rotary shaker at room temperature (see **Note 2**).

2. Wash the seeds five to six times with sterile water under aseptic conditions (in a laminar flowhood) and soak in autoclaved water overnight.
3. Wash the presoaked seeds and remove the seed coat using a scalpel and bent-ended sharp forceps.
4. Place the dehusked seeds on Murashige and Skoog's basal medium supplemented with 3% sucrose and 0.8% agar in a Petri dish (90 × 15 mm) and incubate under standard culture conditions of a 16-h photoperiod at $25 \pm 1^\circ\text{C}$ and a light intensity of $60 \mu\text{E}/\text{m}^2/\text{s}$.
5. Excise primary leaves with their petiolar region intact from 4- to 5-d-old aseptically grown seedlings and cocultivate with *Agrobacterium* culture

3.2. *Agrobacterium* Inoculum Preparation and Cocultivation

1. Inoculate a single colony of *Agrobacterium tumefaciens* strain C58 harboring the binary plasmid pCambia1301 in 25 mL of YEB media containing 50 mg/L kanamycin and incubate at 28°C on a shaker at 100 rpm for 16 to 18 h. Use the culture at late log phase (OD of 0.6 at A_{600}) for cocultivation.
2. Centrifuge the culture at 9000g, discard the supernatant, and dissolve the pellet in 20 mL of half-strength MS liquid medium.
3. Put the cell suspension in a sterile Petri dish (3 mL) by placing in a slanted position with support from the other sterile Petri dish, so the suspension settles at the base of the Petri dish.
4. Dip the petiolar end of the freshly cut leaf explants into the suspension culture for few seconds for infection, blot on sterile filter paper (see **Note 3**), and place them on shoot induction medium (SIM) with their abaxial side in contact with the medium (see **Note 4**) for 2 d at $26 \pm 1^\circ\text{C}$ under continuous cool white light provided by fluorescent lamps ($60 \mu\text{E}/\text{m}^2/\text{s}$) (**Fig. 1A**).

3.3. Shoot Induction, Selection, and Elongation

1. After 2 d, transfer them to fresh SIM medium supplemented with filter-sterilized cefotaxime (250 mg/L) to arrest the growth of *Agrobacterium* (16-h photoperiod at $25 \pm 1^\circ\text{C}$ and a light intensity of $60 \mu\text{E}/\text{m}^2/\text{s}$). Cefotaxime at 250 mg/L is included in all the media during subsequent subcultures until all the *Agrobacterium* overgrowth is arrested.
2. Observe for shoot bud initiation from the cut end of the leaf (**Fig. 1B**) and transfer leaves to half-strength shoot development medium (SDM) after removing half the lamina of the leaf (**Fig. 1C**). Shoot bud initiation can be observed within 7 to 10 d.
3. At this stage supplement SDM medium with 2 mg/L hygromycin as an initial selection pressure to select the transformed shoot from infected culture. With a gap of 7 d (**Fig. 1D**), transfer the explants to the same media with increased concentrations of hygromycin such as 6, 8, and 10 mg/L.
4. Maintain the explants in SDM with frequent subculture for 10 to 21 d (**Fig. 1E** and **F**). Transfer the explants with differentiated and proliferated multiple shoot buds to culture tubes (25 × 150 mm) for elongation in shoot elongation medium (SEM) and maintain for 7 to 14 d (**Fig. 1G**).

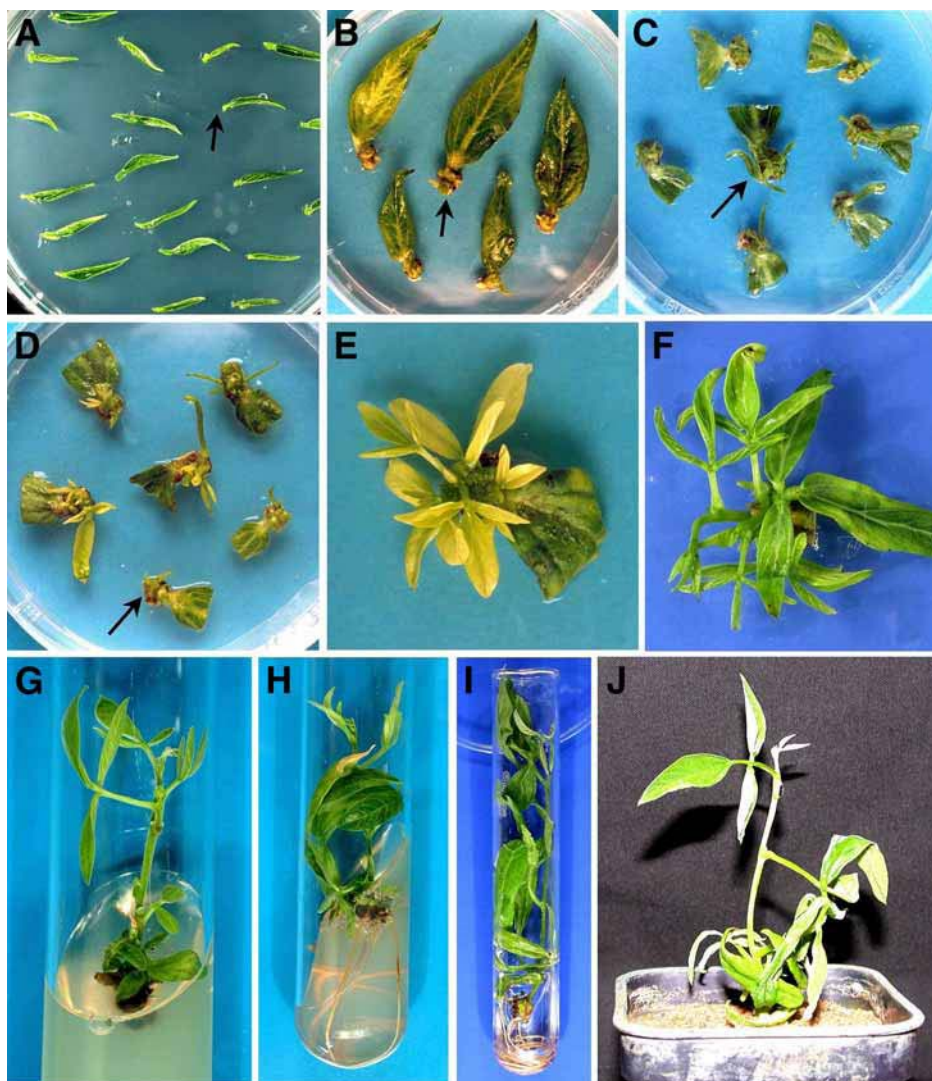


Fig. 1. Regeneration of adventitious shoots and development of plants from leaf explants of *Cajanus cajan* L. (Mills.). Arrows indicate the petiolar cut end undergoing shoot bud differentiation. **(A)** Leaf explants at 0 d on MS medium supplemented with 5 μ M BA and 5 μ M kinetin. **(B)** Differentiation of shoot buds from petiolar cut end. **(C)** Leaf explants with proliferating multiple shoots on shoot development medium with half-cut lamina. **(D)** Development of shoot buds into shoots on reduced SIM. **(E)** Formation of multiple shoots from the petiolar region after 18 d. **(F)** Formation of multiple shoots from the petiolar region after 25 d. **(G)** Individual shoot on MS medium supplemented with 0.58 μ M GA₃ (elongation medium) after 7 d. **(H)** Elongated shoot in rooting media, MS basal with 1% sucrose, after pulse treatment after 7 d. **(I)** Putative transformants showing profuse rooting on MS basal with 1% sucrose after 14 d. **(J)** Well-rooted putative transformant in a 3-inch pot after hardening.

3.4. Rooting

1. Transfer the shoots longer than 3 cm to root induction medium (RIM) with reduced concentration of sucrose (1%) after giving a pulse treatment, dipping the elongated shoots directly into a 11.42 μ M IAA solution for 30 to 60 s. Root initiation can be observed in 7 d (**Fig. 1H**), and upon subculture profuse rooting can be observed within 14 d (**Fig. 1I**).
2. The shoots that are not yet rooted can be given a second pulse treatment with IAA.

3.5. Hardening and Acclimatization of the Plants

1. Gently remove well-rooted plants and wash under tap water to remove media attached to the roots (see **Note 5**).
2. Transfer the regenerated plantlets with well-developed roots to 3-inch (7.62-cm) pots containing autoclaved sand and red soil in a 1:1 ratio for hardening. Every independent plant transferred should be numbered separately and should keep the same numbering in subsequent generations (T_1 , T_2 , and so on).
3. Cover the plantlets with polyethylene bags. This helps the plantlets to minimize loss of water through transpiration and maintains high humidity conditions.
4. Place the plants at 26°C and relative humidity of 40% for 1 d in a growth cabinet, and later transfer them to a greenhouse.
5. After transfer to the greenhouse, punch small holes in the sides of the polyethylene bag and after 3 d remove the top portion of the polythene bag by cutting; eventually remove the bag after 7 to 8 d. This helps the plant to withstand the sudden change in the atmosphere and get acclimatized (**Fig. 1J**).

3.6. Transplantation

1. Transfer the plants from smaller to larger pots (9.5 inches; 24.13 cm) containing autoclaved sand and red soil in a 1:1 ratio supplemented with a small amount of manure and di-ammonium phosphate (DAP).
2. Cover the plants with the same polythene bags with the top portion cut and provide support by placing small rods on either side.
3. After 3 d, uncover the plants totally, expose to withstand various climatic conditions, and maintain the plants in the same conditions till harvest. Depending on the variety used, it takes about 70 to 80 d to flower and 90 to 110 d to maturity; the yield is about 200 to 300 seeds per plant.

3.7. Screening of Transgenics

Independent transgenic plants transferred to the greenhouse are subjected to molecular analysis to check integration and expression of the transferred gene.

3.7.1. Histochemical GUS Analysis

Perform the GUS assay for all plants in all generations to check expression of the *uidA* gene.

1. Take 300 to 500 μL of assay mixture in a 1.5-mL microfuge tube.
2. Cut the leaves from regenerated and hygromycin-resistant plants and incubate in the assay mixture at 37°C overnight in the dark by wrapping in aluminum foil (see **Note 6**).
3. After staining, soak the tissue in 70 and 100% ethanol to clear the chlorophyll content, and finally fix in 70% ethanol until a blue color develops.
4. Observe under the light microscope and photograph.

3.7.2. Characterization of Transgenic Plants

1. To check for integration, isolate the genomic DNA separately using the cetyltrimethylammonium bromide (CTAB) protocol (**15**), digest with the restriction enzyme that has a single recognition site in the plasmid, resolve on 0.8% agarose gel, transfer onto nylon membrane (Hybond N+, Amersham) using a standard protocol, and probe the blot with labeled (radioactive or nonradioactive) PCR-amplified fragments of the *hpt* gene.
2. Carry out genetic analysis to ascertain the inheritance pattern of the introduced genes in the T_1 generation (progenies of primary transformants). This is done by growing all the progenies of the selected events (primary positively confirmed transformant) and then carrying out PCR analysis on the genomic DNA for the introduced genes. The segregation of a single-copy insert usually follows a Mendelian inheritance pattern of 3:1 (i.e., three positives to one negative progeny). In the case of pigeonpea, selection of the seedlings on hygromycin- or kanamycin-containing medium is not very reliable.

4. Notes

1. The assay mixture can be stored for several months at 0°C in a dark bottle.
2. The longer the time in ethanol and mercuric chloride solution lesser the germination efficiency.
3. Care should be taken that only the petiolar region is dipped into the culture; otherwise it becomes difficult to control bacterial growth and infection in the other cut parts that occur during explant preparation.
4. Orientation of the explant on the culture medium is one of the critical factors for obtaining higher regeneration.
5. Washing reduces the chance of bacterial and fungal contamination that may kill the plantlet in soil.
6. The enzyme glucuronidase cleaves the substrate X-Gluc into glucuronic acid and an indoxyl derivative, which dimerizes and oxidizes to form insoluble, highly colored indigo dye.

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Red Clover (*Trifolium pratense*)

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Summary

Genetic modification of plants by the insertion of transgenes can be a powerful experimental approach to answer basic questions about gene product function. This technology can also be used to make improved crop varieties for use in the field. To apply this powerful tool to red clover, an important forage legume, a population of red clover with a high potential for regeneration in tissue culture has been developed. Here we provide a detailed procedure for *Agrobacterium*-mediated transformation of genotypes derived from this regenerable population. We have successfully used this methodology to express a β -glucuronidase (GUS) reporter gene and to silence an endogenous polyphenol oxidase gene in red clover.

Key Words: Genetic transformation; red clover; *Trifolium pratense*; forage legume; posttranscriptional gene silencing.

1. Introduction

Transgenic plants can be extremely powerful research tools. For example, gene expression patterns and protein localization studies can be carried out using reporter genes (1); expression of endogenous genes can be altered (enhanced, ectopic, or reduced) to test hypotheses regarding gene product function; and expression of foreign genes can be used to develop model systems (2). Additionally, genetic transformation of crop plants can allow the introduction of traits that would be difficult to achieve using conventional plant breeding approaches.

Red clover (*Trifolium pratense*) is a widely used and versatile forage legume. To take advantage of the opportunities of genetic transformation for this forage crop, a system of regeneration of red clover plants from transformed plant cells via somatic embryogenesis has been developed (3). Since the ability of callus tissue to regenerate plants has been shown to be under genetic

control in many plant species including forage legumes (4,5), a crucial first step for this system was the development of red clover germplasm with increased frequency of regeneration of whole plants from callus tissue in culture (6,7). Although this population exhibits variability of regeneration response, we have identified several genotypes that have very high potential for regeneration in culture and *Agrobacterium*-mediated transformation. The transformation procedure of these selected genotypes is similar to that of many other *Agrobacterium*-mediated transformation protocols. Petiole explants are infected with *Agrobacteria* harboring a binary vector-based transformation construct consisting of a selectable marker gene and other desired transgenes in the T-DNA region. Following cocultivation, explants are placed on a series of selective media with hormone compositions to stimulate callus formation, embryo induction, and embryo development to plantlets. Plantlets are then placed in a medium to promote rooting prior to transfer to soil. Although the original procedure (3) utilized aseptically grown red clover as the explant source, modifications detailed in this chapter allow the use of greenhouse-grown material, which is easily maintained and provides abundant amounts of explant material with little lead time.

Transformation efficiencies are quite variable and highly genotype dependent (3). For genotypes that perform well, at least 20 to 70% of explants develop selection-resistant callus, with about half of these transformation events going on to develop rooted plantlets. Analysis of expression of a marker-linked β -glucuronidase (GUS) reporter gene indicates that most (at least 70%) selection-resistant callus tissue carry the linked transgene as well (3; M. Sullivan, unpublished data). Overall, the average transformation efficiency using the procedure detailed in this chapter is >10%, with efficiency defined as percent of explants producing fully regenerated, viable plants expressing one or more transgenes. The relative ease of producing large amounts of explant material and manipulating it in culture make transformation of genotypes with even relatively poor (e.g., 1–10%) transformation and regeneration efficiencies tractable.

To date, we have used this procedure to express a GUS gene (3) and to silence an endogenous red clover polyphenol oxidase (PPO) gene (M. Sullivan, unpublished data). Expression of a mannopine synthase promoter-driven GUS gene was stable through sexual transmission (3). In the case of reducing endogenous PPO expression, use of posttranscriptional gene silencing is highly effective in red clover, with all kanamycin-resistant plants tested having 90 to 100% reductions in PPO enzyme activity. These successes indicate that production of transgenic red clover will be an extremely usefully technology in efforts to improve this forage crop.

2. Materials

2.1. Plasmids, *Agrobacterium tumefaciens* Strain, and Plant Material

1. The pART27 binary vector (8) can be obtained from CSIRO Plant Industry (www.pi.csiro.au). Other binary vectors with similar properties, including pMON505 (9), are also available from various sources (10).
2. pRK2013 is available from American Type Culture Collection (www.atcc.org, stock no. 37159).
3. *A. tumefaciens* strain EHA101 (10) can be obtained from Elizabeth Hood (ehood@astate.edu).
4. Several red clover genotypes for transformation selected from a population with superior regeneration in tissue culture (NewRC [6,7]) are available from the authors (see Note 1).

2.2. Solutions

2.2.1. Disinfection Solutions

1. 70% Ethanol: mix 140 mL ethanol with 60 mL ddH₂O.
2. 1% (w/v) Sodium hypochlorite/0.05% (v/v) Tween-20 solution: mix 32 mL household bleach (e.g., Clorox; see Notes 2 and 3), 168 mL ddH₂O, and 100 μ L Tween-20 (polyoxyethylenesorbitan monolaurate, e.g., Sigma P-7949).
3. Sterile ddH₂O: prepare 1 to 2 L for rinsing explants by autoclaving 500-mL aliquots at 121°C for 20 min.

2.2.2. Media Stock Solutions and Components

1. Components to make bacterial and plant culture media including basal salt mixtures, vitamins, antibiotics, hormones, and gelling agents can be purchased from any of several vendors (see Note 4). Timentin (GlaxoSmithKline, Research Triangle Park, NC) can often be purchased from a veterinarian school pharmacy as sterile powder (see Note 5).
2. Prepare stock solutions of antibiotics by dissolving in the appropriate solvent to give the final concentrations indicated in Table 1 (see Note 6), and, if required, filter-sterilizing using a 0.2- μ m syringe filter (e.g., ISC Bioexpress, Kaysville, UT, cat. no. F-2975-5). Store small (1 mL or less) aliquots of antibiotics at -20°C, at which they are stable for at least 1 yr.
3. Prepare stock solutions of α -naphthaleneacetic acid (NAA), kinetin, 2,4-dichlorophenoxyacetic acid (2,4-D), and adenine (not adenine sulfate) as indicated in Table 2. For each, dissolve the hormone in the indicated solvent (if required), bring to the indicated final volume with ddH₂O, and filter-sterilize. Divide NAA, kinetin, and adenine stock solutions into 5- to 10-mL aliquots, and store at -20°C, at which they are stable for over 1 yr. Store 2,4-D stock solution at 4°C, at which it is stable for at least several months (see Note 7).

Table 1
Antibiotic Stock Solutions

Antibiotic	Stock	Concentration (mg/mL) ^a	Solvent	Sterilization
Kanamycin sulfate	1000X	50	ddH ₂ O	Filter
Spectinomycin	1000X	100	ddH ₂ O	Filter
Rifampicin	1000X	25	DMSO	None
Timentin	500X	250	ddH ₂ O	Filter

^aSee **Note 6**.

Table 2
Hormone Stock Solutions

Hormone	Concentration (mg/mL)	Amount (mg)	Solvent	Diluent
NAA	2.00	100	800 μ L 1 N NaOH	ddH ₂ O to 50 mL
Kinetin	2.12	53	400 μ L 1 N NaOH	ddH ₂ O to 25 mL
2,4-D	1.00	50	5 mL Ethanol	ddH ₂ O to 50 mL
Adenine	2.00	50	—	ddH ₂ O to 25 mL

2.3. Media

2.3.1. Bacterial Media

1. LB medium: dissolve 10 g tryptone, 10 g NaCl, and 5 g yeast extract in 1 L ddH₂O; adjust pH to 7.2 with 1 N NaOH; dispense into convenient volumes (for liquid medium); and autoclave at 121°C for 20 min. For solid medium, add 15 g/L agar prior to autoclaving. Cool solid media in a 55°C water bath, add antibiotics (*see* **Note 8**), and dispense media into sterile 15 $\times$ 100-mm disposable Petri dishes. Liquid media without antibiotics are stable at room temperature for at least 1 yr. Solid media with antibiotics are stable for at least 4 to 6 wk when stored at 4°C.
2. YEP medium: dissolve 10 g peptone, 10 g yeast extract, and 5 g NaCl in 1 L ddH₂O; dispense into convenient volumes (for liquid medium); and autoclave at 121°C for 20 min. Solid YEP medium preparation and stability is as described above for LB medium.
3. 30% Glycerol solution (freezer medium): dissolve 30 mL glycerol in 70 mL of ddH₂O and autoclave at 121°C for 20 min.

2.3.2. Plant Media

1. Gamborg's B5-based media (**11**) is used throughout the transformation protocol. Components to make 1 L of media are listed in **Table 3**. To prepare any of these

Table 3
Red Clover Transformation Media Components to Make 1 L

Component	B5 Wash	CIM	CIM-KT	EIM-KT	EDM-KT	RM-KT
B5 basal salts	3.08 g	3.08 g	3.08 g	3.08 g	3.08 g	3.08 g
Sucrose	20 g	20 g	20 g	20 g	20 g	20 g
Vitamins						
Myoinositol	—	100 mg	100 mg	100 mg	100 mg	100 mg
1000X Vitamins ^a	—	1 mL	1 mL	1 mL	1 mL	—
Thiamine-HCl	—	—	—	10 mg	10 mg	—
Phytagar ^b	—	7.0 g	7.0 g	7.0 g	7.0 g	7.0 g
Hormones ^c						
NAA	—	2.00 mg	2.00 mg	2.00 mg	0.20 mg	—
2,4-D	—	2.25 mg	2.25 mg	—	—	—
Kinetin	—	2.12 mg	2.12 mg	—	—	—
Adenine	—	—	—	2.00 mg	—	—
Antibiotics ^c						
Kanamycin	—	—	50 mg	50 mg	50 mg	50 mg
Timentin ^d	—	—	500 mg	500 mg	500 mg	500 mg

^aSee **Note 9**.

^bSee **Note 4**.

^cMany, but not all of the hormone and antibiotic stock solutions detailed in the text are 1000X. Make sure to calculate the required amount of any stock to give the desired final concentration. Especially noteworthy are 2,4-D (use 2.25 mL of the 1 mg/mL stock), NAA in EDM-KT (use 100 µL of the 2 mg/mL stock), and timentin (use 2 mL of the 250 mg/mL stock).

^dSee **Note 5**.

- media, sprinkle Gamborg's B5 basal salts over approximately 900 mL ddH₂O while rapidly stirring to dissolve. Add sucrose and any required vitamins (*see Note 9*). Adjust the pH of the media to 5.8 with 1 N KOH. The buffering capacity of these media is not very high, so add KOH dropwise to avoid overshooting pH 5.8.
2. Adjust volume to 1 L with ddH₂O. For B5 wash medium, dispense working aliquots (e.g., 200 mL) into bottles. For the solid media, transfer to a 2-L flask, add the Phytagar (or other gelling agent; *see Note 4*), and cover the flask with foil. Autoclave the media for 20 min at 121°C.
 3. Following sterilization, place the flask(s) containing solid media in a 55°C water bath.
 4. When media have cooled, working in a laminar flow hood or biological safety cabinet, add hormone and antibiotic stock solutions to achieve the desired final concentrations, and mix thoroughly (*see Note 8*).
 5. Dispense the media into sterile containers as follows: CIM, 100 × 15-mm Petri plates (30–40 plates per L); CIM-KT, EIM-KT, and EDM-KT, 100 × 20-mm Petri plates (20–30 plates per L); and RM-KT, Magenta GA7 lidded vessels (15–20 boxes per L).
 6. Allow the media to solidify and dry out slightly. Leaving fresh media in a closed hood (not running) for a few days allows it to dry out slightly and makes it easier to use and store.
 7. If media are not used within a few days, package plates and Magenta boxes in the plate sleeves or other plastic bags and store at 4°C (*see Note 10*). Use media with hormones and antibiotics within 1 mo. B5 wash medium can be stored for several months at room temperature.

2.4. Additional Supplies

1. Commercially available plasmid isolation kits (e.g., QIAprep Spin Miniprep Kit, Qiagen, Valencia, CA) can be used for preparing DNA from *Agrobacterium* or *E. coli*.
2. A variety of standard plant tissue culture tools (forceps, scalpels, and so on) are required for manipulating tissue explants; 15-cm bent-tip forceps are especially useful for the required tissue manipulations.
3. If the optional wash step will be carried out, 7.5-cm steel kitchen strainers (one for each construct to be transformed) and several (two for each construct to be transformed) 9-cm² pieces of thick filter paper (e.g., Whatman 3MM; Whatman, Clifton, NJ) should be wrapped in double layers of aluminum foil and sterilized by autoclaving at 121°C for 20 min.
4. One-inch Micropore tape (3M Corporation, St. Paul, MN; available from many plant tissue culture and medical supply companies) or Parafilm M (Pechiney Plastic Packaging, Chicago, IL; available from many scientific supply companies) is used for sealing tissue culture plates.

3. Methods

3.1. Preparation of Greenhouse-Grown Plant Material for Transformation

1. Maintain red clover plants, one plant per 10- to 15-cm pot, in a standard commercial potting mix (e.g., Pro-Mix BX, Premier Horticulture, Quakertown, PA) and fertilize weekly with an all-purpose fertilizer (e.g., Peter's soluble 20-20-20, Scott's, Marysville, OH) according to the manufacturer's instructions. Maintain the greenhouse temperature between 20 and 30°C with light intensities between 400 and 1000 $\mu\text{mol}/\text{m}^2/\text{s}$. Use supplemental lighting when day length is less than 15 h/day. Every 1 to 3 mo, cut back red clover to 5 cm above the soil to promote new growth.
2. Clonally propagate red clover by splitting off pieces of crown with an attached root, dipping in a commercial rooting powder with fungicide such as Rootone (GardenTech, Lexington, KY; available from nursery and garden supply centers), and transferring to moist potting mix in a 5- to 8-cm pot. Keep the rooting plants well watered in a growth chamber at approximately 25°C constant temperature with 15 to 16 h per day of approximately 100 $\mu\text{mol}/\text{m}^2/\text{s}$ illumination from fluorescent lamps until established. Plants may be transferred to the greenhouse with appropriate acclimation to the higher (i.e., 400–1000 $\mu\text{mol}/\text{m}^2/\text{s}$) light conditions. We often place plants under a mesh screen to reduce light intensity for the first few days following transfer to the greenhouse.
3. Plants are treated as needed by application of a fungicide such as Cleary's 3336F (dimethyl 4,4'-*o*-phenylenebis[3-thioallophanate]; Cleary Chemical Company, Dayton, NJ), available from greenhouse and nursery supply companies. This systemic fungicide is used as a soil drench according to the manufacturer's instructions (see **Note 11**).

3.2. Preparation of Transformation Constructs and *Agrobacterium tumefaciens* Strains

1. Prepare a transformation construct in a suitable binary vector using standard molecular biology techniques. The transformation method described in this chapter has been used for constructs made in pART27 (**8**) and pMON505 (**9,12**) utilizing an *nptII* selectable marker driven by the nopaline synthase promoter (see **Note 12**) with successful expression of linked transgenes from mannopine synthase and CaMV 35S promoters (**3**; M. Sullivan, unpublished data).
2. Introduce the binary vector construct into *A. tumefaciens* strain EHA101 (see **Note 13**) by triparental mating (see **Note 14**) mediated by pRK2013 described in Chapter 3 and elsewhere (**13**), with selection on solid LB medium supplemented with appropriate antibiotics (e.g., for pART27-based constructs in EHA101, use rifampicin [25 mg/L], kanamycin [50 mg/L], and spectinomycin [100 mg/L]).
3. Inoculate two to four single colonies resulting from the mating into separate culture tubes containing 3 mL LB or YEP medium supplemented with appropriate antibiotics. Grow for 1 to 2 d at 28°C with shaking (200 rpm).

4. Prepare plasmid from 2 mL of the culture using a commercial plasmid miniprep kit or as described in Chapter 5 and confirm its identity by carrying out appropriate restriction digestions. Direct digestion of the *Agrobacterium*-derived DNA preparation often gives satisfactory results (see **Note 15**). If results are not satisfactory, the *Agrobacterium*-derived plasmid can be transformed into a suitable *E. coli* strain (e.g., XL-1 Blue, DH5 α , JM101) for preparation of higher quality DNA. In either case, sufficient analysis of the transferred plasmid should be carried out to ensure that no rearrangements have taken place.
5. Prepare a glycerol stock of the remaining culture from **step 3** by mixing 600 μ L *Agrobacterium* culture with 300 μ L sterile 30% glycerol in a microcentrifuge tube. This stock can be stored at -70°C indefinitely.
6. At least 3 d prior to plant transformation, streak out the *A. tumefaciens* culture (from the glycerol stock) on solid LB medium supplemented with appropriate antibiotics (e.g., for pART27- or pMON505-derived plasmids in EHA101 use spectinomycin and kanamycin, but not rifampicin; see **Note 16**). Incubate at 28°C for 2 d until colonies appear. Although the *Agrobacterium*-containing plates can be kept at 4°C for several weeks, liquid cultures for cocultivation with explants (**step 7**, this section) tend to grow more predictably from fresh plates.
7. One day prior to plant transformation, inoculate YEP medium supplemented with appropriate antibiotics (i.e., as in **step 2** above, but rifampicin selection is not required) with *A. tumefaciens* from the plate in **step 6**. Approximately 1 mL of culture will be needed to infect 100 petiole explants. Grow overnight at 28°C with shaking at 200 rpm until the cultures are near the end of logarithmic growth ($\text{OD}_{600\text{nm}}$ of approx 1.5). It is often convenient to start more than one culture with different amounts of inoculum to achieve the desired growth overnight. The resulting culture will be used directly for the transformation.

3.3. Preparation of Explant Material from Greenhouse-Grown Plants

1. One week prior to transformation, if desired, treat the greenhouse-grown plants with a systemic fungicide such as Cleary's 3336F, as a soil drench according to the manufacturer's instructions (see **Note 17**).
2. On the day of the transformation, harvest the petioles of young leaves from healthy-looking plants. Select petioles that are <6 cm in length (see **Note 18**). Use a razor blade to cut each petiole from the plant, and remove its leaves.
3. Working in a laminar flow hood or biological safety cabinet, surface-sterilize batches of 5 to 10 intact petioles in a series of five 15×150 -mm sterile Petri dishes containing the indicated solutions (approximately 100 mL/plate) as follows: 70% ethanol for 1 min; 1% (w/v) sodium hypochlorite/0.05% (v/v) Tween-20 for 1 min; three successive sterile ddH $_2$ O rinses.
4. Use sterile forceps (see **Note 19**) to agitate the petioles gently in each solution. Ensure that tissues become thoroughly wetted with each solution and that no air bubbles cling to the tissue surface. Ethanol and sodium hypochlorite can be used repeatedly and replenished in the Petri dishes as needed. Sterile ddH $_2$ O for rinses should be changed after every two batches of petioles.

5. As each batch of petioles is surface-sterilized, leave them in the final ddH₂O rinse and proceed to the first steps of transformation procedure (see **Subheading 3.4.**) before sterilizing another batch.

3.4. Transformation Procedure

As above, carry out all tissue culture procedures in a laminar flow hood or biological safety cabinet. Carry out all red clover tissue culture incubations at 26°C with 16 h per day of approximately 40 $\mu\text{mol}/\text{m}^2/\text{s}$ illumination from fluorescent lamps (see **Note 20**).

1. Transfer harvested, surface-sterilized petioles (see **Subheading 3.3.** above) to a sterile 15 $\times$ 150-mm Petri dish. Work with one or two petioles at a time to prevent them from becoming desiccated.
2. Using a sterile scalpel and forceps, cut each petiole into 4- to 6-mm pieces. Place 20 to 30 of these pieces onto CIM (no antibiotics), spacing them evenly across the plate surface. The sections do not have to be embedded in the medium. Once a plate is filled with explants (and when you are not working with a given plate), cover with the lid to prevent the tissue and medium from drying out. Continue harvesting and sterilizing petioles, cutting into sections, and placing on plates until the desired number of plates/explants is achieved. Prepare two to four plates of explants for each transformation construct and two to four additional plates of explants if controls for selection and plant regeneration (described in more detail in **steps 3** and **6** below) will be carried out.
3. Infect the petiole explants with the *Agrobacterium* cultures (from **step 7** of **Subheading 3.2.** above) by using a pipetor to add a drop of culture directly to each explant on the plate. Make sure the drop surrounds the explant. Use only one *Agrobacterium* strain per plate. If carrying out controls for selection and plant regeneration, leave two to four plates of explants uninfected.
4. Allow the culture to soak into the plate for a few minutes, and then seal all the way around the plate with 3M Micropore tape or Parafilm (see **Note 21**).
5. Incubate the plates for 48 h. During this period, the *agrobacteria* will overgrow the explant pieces.
6. Transfer up to 100 explant pieces to a sterile 50-mL tube containing 35 mL of B5 wash medium. (Do separate washes for each different transformation construct.) Gently shake the tube to dislodge excess bacteria. Drain into a sterile 7.5 cm kitchen sieve placed above a beaker, and then dump the explants onto a 9-cm square of sterile 3 MM filter paper in a sterile 15 $\times$ 150-mm Petri dish. Blot the explants with a second piece of 3 MM filter paper. Use sterile forceps to transfer the washed (see **Note 22**) explants to fresh CIM-KT. Push each explant slightly into the medium to embed it partially. At this stage, transfer the explants from half the uninfected control plates to CIM medium with timentin but lacking kanamycin (see **Note 23**) as a control for plant regeneration. The remaining uninfected explants can be transferred to CIM-KT as a control for kanamycin selection. Seal the plates with Micropore tape or Parafilm and return them to the incubator or growth room.

7. Check plates every other day for contamination. When using greenhouse-grown tissue, it is not uncommon to see some explants with fungal or bacterial contamination in the first few weeks following introduction into tissue culture. If contamination is discovered, transfer noncontaminated explants to a fresh CIM-KT plate. Make sure to resterilize tools often when transferring explants. Frequent sterilization of tools will avoid spreading undetected contamination.
8. Two to 3 wk following *Agrobacterium* cocultivation, callus formation on the explants will become apparent. For *Agrobacterium*-infected explants on selective media, the callus will generally begin forming on the cut ends. For control explants on nonselective media, callus tends to form over the length of the explant. Transformation efficiency is highly variable and genotype dependent. With continued incubation on CIM-KT, we generally see 20 to 80% of *Agrobacterium*-infected explants form callus on selective media for our selected genotype NewRC27. Nearly all explants on control plates without selection will show extensive callus development. Explants not infected with *Agrobacterium* should show little if any callus formation on kanamycin-containing medium. Experiments with a marker-linked GUS reporter gene indicate that most (>70%) callus formation on selective medium represents an actual transformation event.
9. After a total of 4 to 5 wk of incubation on CIM-KT, transfer explants to EIM-KT, pressing them slightly into the medium, and seal the plates with Micropore tape or Parafilm. For the regeneration control (i.e., no selection), transfer explants to EIM with timentin only (see **Note 23**). Within 1 to 2 wk, green embryos should begin to form (see **Note 24**). Continue incubation on EIM-KT. Unless a plate shows signs of contamination, there is no need to transfer explants to fresh media during this incubation.
10. After a total of 4 to 5 wk on EIM-KT, transfer the forming clumps of green embryonic tissue to EDM-KT, pressing them slightly into the medium. Independent transformation events should be kept separated. Embryos derived from different explants are certainly independent transformation events, and often tissues derived from opposite ends of an explant are independent events (see **Note 25**). As multiple plantlets will ultimately be derived from some of the tissue at this step, we number the tissue clumps (on the bottom of the Petri plate) to aid in keeping track of independent events. Seal the plates with Micropore tape or Parafilm and return them to the incubator or growth room.
11. In approximately 3 to 5 wk, distinct plantlets with trifoliate leaves will begin to form. As vigorous healthy plantlets form, gently remove them using forceps (and if necessary a scalpel), and insert the basal end into RM-KT in a Magenta vessel. Four to five plantlets can be placed in a single vessel. Track the tissue clump (i.e., independent event) from which each plantlet is derived by transferring its number to the bottom or side of the Magenta vessel. Transfer multiple nonindependent transformants to RM-KT, since some may not root or may be otherwise lost to contamination. Continue to incubate the EDM-KT plates, as plantlets may continue to form over the next 4 to 8 wk. Transfer the tissues to a fresh EDM-KT plate every 4 to 6 wk, if necessary.

12. Incubate the plantlets on RM-KT for several weeks. Rooting time is quite variable. Some plantlets may begin rooting in less than 1 wk, and most will root within 3 to 4 wk.
13. When a vigorous root system has formed, transfer plants to soil (*see Note 26*). Gently pull each plantlet from the rooting medium, remove excess medium from its roots by gently washing with tap water, and plant in moist sterile potting mix (e.g., Pro-Mix BX) in a 5- to 8-cm pot. Immediately place the potted plant in a 1-gallon food storage bag, and close loosely with a twist tie.
14. Place the bagged, potted plants in a growth chamber at approximately 25°C constant temperature with 15 to 16 h per day of approximately 100 $\mu\text{mol}/\text{m}^2/\text{s}$ illumination from fluorescent lamps. To acclimate the plants to lower humidity conditions, make a few 1-cm slits in the plastic bags after 1 to 2 d. Increase the number and size of the slits every 1 to 2 d. Plants are acclimated to growth chamber conditions after 7 to 10 d and can be removed from the plastic bags. We see >90% survival when plants with well-developed root systems are transferred to soil

3.5. Maintaining Transgenic Red Clover

1. Once plants are established, avoid overwatering. Allow the soil to dry out between watering.
2. Fertilize every 2 to 3 wk with an all-purpose fertilizer such as Peter's 20-20-20.
3. Periodic treatment (every 1–2 mo) with an antifungal agent such as Cleary's 3336F may help prevent loss to fungal diseases (*see Note 11*).
4. Plants may be grown under greenhouse conditions following appropriate acclimation (described in **Subheading 3.1.** above) to the higher (i.e., 400–1000 $\mu\text{mol}/\text{m}^2/\text{s}$) light conditions. Take appropriate containment precautions for transgenic plants as may be required by local and/or national regulations (*see Note 27*).
5. Transgenic red clover can be clonally propagated as described in **Subheading 3.1.** above.
6. Sexual hybridization with nontransgenic plants by standard methodologies (**14**) is successful. A mannopine synthase promoter::GUS transgene was expressed in the expected 1:1 ratio in progeny, and all GUS-positive plants were positive for the *ntpIII* marker gene (**3**).

4. Notes

1. Some of these selected genotypes are available from the authors upon request. Alternatively, additional transformable genotypes could be identified from NewRC germplasm (**6,7**) by growing 25 to 50 plants from seed. Three to five of the best regenerating plants could be identified by following the regeneration protocol outlined in this chapter (omitting *Agrobacterium* infection and marker selection, if desired). Highly regenerable genotypes thus identified could then be candidates for transformation.
2. Mention of trade names, commercial products, or specific vendors in this chapter is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

3. Household bleach can vary in sodium hypochlorite content. The amounts given here are for household bleach containing 6.25% sodium hypochlorite. Make sure to check the product label and adjust dilution to obtain a 1% (w/v) final concentration of sodium hypochlorite. The bleach/tween solution should always be freshly prepared to prevent hypochlorite loss.
4. Vendors that sell plant tissue culture media and supplies include Sigma (St. Louis, MO), Phytotechnology Laboratories (Shawnee Mission, KS), and Caisson Laboratories (Rexburg, ID). Gibco BRL, now Invitrogen (Carlsbad, CA), has discontinued most of their plant tissue culture reagents, including Phytagar, which we have used in all our experiments to date. Phytablend Agar (Caisson Laboratories) is, according to the vendor, comparable to Gibco BRL Phytagar, although we have not yet tested it with the red clover tissue culture system.
5. The original protocol (3) used 300 mg/L carbenicillin to select against *Agrobacterium* following cocultivation. Timentin at 500 mg/L is more effective at selecting against *Agrobacterium* and/or endogenous bacterial contaminants and does not interfere with plant regeneration.
6. For kanamycin sulfate, spectinomycin, and rifampicin, the volume contribution of the antibiotics themselves to the final solution is small and can be ignored (i.e., dissolve 50, 100, or 25 mg of each antibiotic, respectively, in each 1 mL of solvent). Because of the relatively high concentration of the timentin stock solution, however, dissolve each 250 mg of this antibiotic in only 870 μ L H₂O to achieve the desired final concentration. Timentin is sometimes supplied as 3.1 g of sterile powder in a vial. In this case, add 10.8 mL sterile ddH₂O directly to the vial to make 12.4 mL of sterile 250 mg/mL timentin solution.
7. Prior to use, check to be sure 2,4-D is dissolved. If crystals are present in the stock solution, warm to room temperature to dissolve. If the crystals do not dissolve, prepare a new stock solution.
8. Mixing can be achieved by adding sterile magnetic stir bars to the media after autoclaving. Gentle stirring following hormone and antibiotic additions avoids frothy media. Do not autoclave stir bars in the media; this frequently results in boil-over and volume loss.
9. Prepared Gamborg's 1000X vitamin stock solution contains (per mL) 10 mg thiamine-HCl, 1 mg pyridoxine-HCl, and 1 mg nicotinic acid in ddH₂O and can be purchased or prepared from individual chemicals. Be sure to check suppliers' compositions carefully, as some sources may include myoinositol as well. Aliquots (5–10 mL) of the vitamin mixture can be stored at –20°C for several years.
10. We usually prepare hormone-containing media 1 to 2 d prior to use. If leftover media are stored, store wrapped plates inverted at 4°C, and look for signs of contamination prior to use.
11. This or any fungicide should be used in conjunction or rotation with another fungicide product having a different mode of action to prevent the development of resistant fungi.
12. We have only carried out the transformation procedure using the *nptII* selectable marker, although we have no reason to believe other selection systems (e.g., hygromycin or basta resistance) cannot be used.

13. Although strong strain X genotype interactions have been observed with this transformation system, *A. tumefaciens* strain EHA101 seems to be generally superior to one of the other tested strains, A280 (3).
14. Other methods of introducing binary vector constructs into *Agrobacterium* can also be used, although we find triparental mating easy, efficient, and reliable.
15. Be aware that cleavage at some restriction sites may differ between DNA derived from *Agrobacterium* and *E. coli* owing to differences in DNA methylation. For example, pMON505 contains Cla I sites that are cleaved in DNA prepared from *Agrobacterium* but not *E. coli*.
16. Because EHA101 strains inoculated directly from a frozen glycerol stock grow poorly on media with rifampicin, these should be streaked, at least initially, onto medium lacking rifampicin. Once cultures begin to grow, restreak them onto medium containing all selective antibiotics to ensure rifampicin resistance, if desired.
17. Although we have not rigorously tested this antifungal pretransformation treatment, preliminary results suggest it may be effective at reducing fungal contamination in culture. Cleary's 3336F does not appear to have an effect on plant regeneration in culture. Although it is not yet clear whether pretreatment with Cleary's 3336F might reduce transformation efficiency, we have been able to recover adequate numbers of transformants following its use.
18. For the NewRC genotypes we have worked with, petiole length seems to be a good indicator of age. Petioles from young leaves (those with petioles < 6 cm) have a much higher transformation efficiency than those of older leaves. We have not tested whether there are any basal/apical effects within an individual petiole.
19. Sterilize instruments by dipping in 70% ethanol and flaming, or by using a glass bead sterilizer. Place working portions of instruments in a sterile Petri dish to allow instruments to cool before use. Care should be taken when working with ethanol solutions in the presence of an open flame.
20. A tissue culture incubator is not required provided appropriate lighting and temperature control can be maintained.
21. We have not compared the efficacy of Micropore tape and Parafilm. Both will work.
22. Although we include a wash step here, it has been successfully omitted (3). If this step is omitted, simply transfer the explants to CIM-KT medium.
23. Only a few plates of media lacking kanamycin are required for the regeneration control. To make these, prepare the media as usual, add all required hormones and timentin, but do not add kanamycin. Dispense 35 mL media into each of a few Petri plates. (A sterile 50-mL conical tube is an easy way to measure this.) Add kanamycin stock solution (take into account the lost volume and adjust accordingly) to the remaining media, stir, and dispense into Petri plates.
24. The effects of selection on the uninfected control explants are usually quite obvious after incubation on CIM-KT, so we often discontinue this control. When we have placed uninfected control explants on EIM-KT, no green embryos have developed.

25. We take a conservative view in considering which transformation events are independent. If there is any doubt, consider all tissue derived from a given explant as nonindependent.
26. If not all plants in a Magenta vessel have rooted, plants to be transferred to soil can be removed aseptically in a laminar flow hood or biological safety cabinet. Work quickly to avoid desiccation of the plantlets.
27. See, for example, *A Practical Guide to Containment* (15), available free of charge at <http://www.isb.vt.edu>.

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Soybean (*Glycine max*) Transformation Using Mature Cotyledonary Node Explants

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Summary

Agrobacterium tumefaciens-mediated transformation of soybeans has been steadily improved since its development in 1988. Soybean transformation is now possible in a range of genotypes from different maturity groups using different explants as sources of regenerable cells, various selectable marker genes and selective agents, and different *A. tumefaciens* strains. The cotyledonary-node method has been extensively investigated and across a number of laboratories yields on average greater than 1% transformation efficiency (one Southern-positive, independent event per 100 cotyledonary-node explants). Continued improvements in the cotyledonary-node method concomitant with further increases in transformation efficiency will enhance broader adoption of this already productive transformation method for use in crop improvement and functional genomics research efforts.

Key Words: Genetic engineering; transgenic; legumes; axillary meristems; hygromycin phosphotransferase; Mendelian inheritance; nuclear transformation.

1. Introduction

Transgenic soybean plants have been produced using microprojectile bombardment of shoot meristems (1) and embryogenic suspension cultures (2,3) and *Agrobacterium tumefaciens*-mediated T-DNA delivery into immature cotyledons (2,4–6), embryogenic suspension cultures (7), shoot meristems (8–10), and axillary meristems located in seedling cotyledonary nodes (11). Although these multiple methods provide researchers with a range of approaches to produce transgenic soybean plants, in general they are constrained by relatively low transformation efficiencies. Thus, continued improvements are necessary for developing robust methods for soybean improvement and for functional genomics studies (12).

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This chapter is focused on the *A. tumefaciens*-mediated cotyledonary-node transformation method originally reported by Hinchey et al. in 1988 (11). Like all *A. tumefaciens*-mediated transformation methods, the cotyledonary-node method depends on targeting T-DNA delivery into regenerable cells, specifically the meristematic tissue of the cotyledonary node, followed by selection for transgenic cell proliferation and shoot formation. In 2001, Olhoft et al. (13,14) demonstrated that inclusion of thiol compounds in the solid cocultivation medium increased T-DNA delivery into the target cells in the cotyledonary node, resulting in increased production of transgenic plants. Another important improvement that has also boosted production of transgenic plants and facilitated a broader adoption of the cotyledonary-node method involved identifying alternative selective agents (15,16) and selection schemes (17). The resultant method is comparatively simple, inexpensive, rapid, and efficient and, most importantly, is now quite reproducible, as indicated by its use in a number of laboratories (13–19). The cotyledonary-node method uses young seedlings for explant preparation, which substantially reduces the costs and labor associated with growing plants for production of immature embryos or maintaining long-term tissue cultures. Moreover, it has been used on a range of cultivars with various *Agrobacterium* strains and binary plasmids. Because it is based on T-DNA delivery, the method has a higher probability of producing simple, nonrearranged transgene loci compared with direct delivery methods (20,21).

For example, we recently evaluated transgene locus structure in a population of 95 independent soybean plants (18). On average, each plant exhibited two T-DNA loci, one of which was a simple locus that exhibited all portions of the T-DNA and no rearranged T-DNA or binary plasmid backbone and was transmitted in a Mendelian manner (18). Beyond increasing the recovery of transgenic plants that exhibit high-fidelity transgene expression and Mendelian transmission of the transgenic phenotype, simple transgene loci will streamline regulatory characterization and eventual commercial development. Although the transformation frequency has been improved to greater than 10% (15), the method requires empirical adjustments of selection regime and other system components for transformation of a broader range of soybean genotypes (17). Therefore, this chapter is intended to provide researchers with a foundation for further improving the cotyledonary-node transformation method.

1.1. Two Cautionary Notes

First, our experiences in adopting and working with the cotyledonary-node method have taught us that it is most efficient (in terms of time, labor, and cost savings) to learn this method from a colleague who has it up and running. It is

nearly impossible to explain all the essential details and subtleties in a chapter format. For the researcher who has no experience with the cotyledonary-node method, acquiring hands-on experience with media and explant preparation is the fastest and cheapest means of establishing the transformation system. On the basis of observation of several researchers who are experienced with other plant transformation systems and attempt to learn the cotyledonary-node system, it appears that application of *a priori* assumptions or shortcuts can be extremely counterproductive. Second, when you are attempting to establish the protocol in your own lab, we recommend strictly following the protocol outlined below or a working method transferred from a colleague. Substituting media components or otherwise departing from the outlined steps may drastically reduce transformation efficiency. Our advice, which is based on more than 20 researcher-years of experience with the cotyledonary method, is to get the method working first and then begin to investigate improvements.

2. Materials

2.1. *A. tumefaciens* Strains

C58, AGL1, EHA101, EHA105, and LBA4404 transformed with a range of binary plasmids have been used for soybean transformation using the cotyledonary node method (7,15–19). The method described here has been optimized for hygromycin B selection using the hygromycin phosphotransferase (*hpt*) gene (15). Binary plasmids carrying *hpt* fused to the cauliflower mosaic virus 35S promoter, such as pGPTV, pTOK233, and pCAMBIA 1303 and 1309, provide excellent selection. They also confer sufficient expression in *A. tumefaciens* to allow selection for the presence of the binary plasmid in the bacteria.

2.2. Plant Materials

Field-grown, disease-free soybean seeds are used for explant preparation. We use the cultivar ‘Bert’, a maturity group III genotype available from the Minnesota Crop Improvement Association.

2.3. Media Stock Solutions

All stocks are made with double-distilled water (ddH₂O) and stored at 4°C unless otherwise noted. Filter-sterilize solutions using a 25-mm syringe with a 0.2-μm size pore Acrodisc® filter when noted.

1. 10X B5 major salts: 250 mM KNO₃, 10 mM CaCl₂, 10 mM MgSO₄, 10 mM (NH₄)₂SO₄, 10 mM NaH₂PO₄.
2. 100X B5 minor salts: 5 mM H₃BO₃, 6 mM MnSO₄, 0.7 mM ZnSO₄, 0.45 mM KI, 0.1 mM Na₂MoO₄, 0.01 mM CuSO₄, 0.01 mM CoCl₂.

3. 100X B5 vitamins: 55 mM myoinositol, 0.8 mM nicotinic acid, 0.5 mM pyridoxine-HCl, 3 mM thiamine-HCl.
4. 10X MS major salts: 20 mM NH_4NO_3 , 200 mM KNO_3 , 30 mM CaCl_2 , 15 mM MgSO_4 , 12.5 mM KH_2PO_4 .
5. 100X MS minor salts: 10 mM H_3BO_3 , 10 mM MnSO_4 , 3 mM ZnSO_4 , 0.5 mM KI, 0.1 mM Na_2MoO_4 , 0.01 mM CuSO_4 , 0.01 mM CoCl_2 .
6. 100X MSIII iron: 10 mM FeSO_4 , 10 mM NaEDTA. Dissolve FeSO_4 in 200 mL ddH₂O and in a separate container, boil EDTA for 2 min in 200 mL ddH₂O. Slowly combine the two solutions and cool to room temperature before bringing the final volume to 1 L.
7. Indole-3-acetic acid (IAA): 1 mg/mL stock solution. Dissolve 20 mg in 20 mL 95% ethyl alcohol. Make 1-mL aliquots and store at -20°C .
8. Gibberellic acid (GA_3): 1 mg/mL stock solution. Dissolve 50 mg GA_3 in 50 mL 70% ethyl alcohol. Store at 4°C .
9. 6-Benzyl-aminopurine (BAP): 1 mg/mL stock solution. Dissolve 100 mg BAP in 0.2 N HCl; bring solution up to 100 mL with ddH₂O. Filter-sterilize. Store at 4°C .
10. trans-Zeatin riboside (ZR): 1 mg/mL stock solution. Dissolve 50 mg ZR in 0.2 N HCl; bring solution up to 50 mL with ddH₂O. Filter-sterilize. Store at -20°C .
11. Indole-3-butyric acid (IBA): 1 mg/mL stock solution. Dissolve 20 mg IBA in 20 mL 95% ethyl alcohol. Store at -20°C .
12. Hygromycin B (Boehringer Mannheim): 50 mg/mL stock solution. Dissolve 1 g hygromycin B in 20 mL ddH₂O. Filter-sterilize. Store at -20°C .

2.4. Culture Media for *A. tumefaciens*

The following recipes use the stock solutions given in **Subheading 2.3.** above and are calculated per liter total volume:

1. Yeast extract peptone (YEP) Medium: 1% Bacto-peptone (Difco), 0.5% yeast-extract (Difco), 0.5% NaCl, adjusted to pH 7.0 with 1 N NaOH, solidified with 1.2% granulated agar (Difco) and poured into 15×100 -mm Petri dishes.
2. Liquid cocultivation medium (LCCM): 1/10 strength B5 major and minor salts, 1/10 MSIII iron, 3% sucrose, and 3.9 g/L 2-[N-morpholino]ethanesulfonic acid (MES), adjusted to pH 5.4 with 10 N KOH. In a separate beaker, dissolve 40 mg acetosyringone in 5 mL 95% ethyl alcohol and add 10 mL 100X B5 vitamin stock, 0.25 μL GA_3 stock, and 1.67 mL BAP stock per liter of basic medium and filter-sterilize. Add the filter-sterilized solution to cooled LCCM.

2.5. Soybean Tissue Culture Media

1. Germination medium (GM): full-strength B5 major and minor salts, MSIII iron, B5 vitamins, 2% sucrose, adjusted to pH 5.8 with 1 N KOH, solidified with 0.8% Noble Agar (Difco), autoclaved, and poured into 25×100 -mm Petri dishes.
2. Solid cocultivation medium (SCCM):
 - a. Autoclave 1/10 strength B5 major and minor salts, 1/10 strength MSIII iron, B5 vitamins, 3.9 g/L MES, 3% sucrose, adjusted to pH 5.4 with 10 N KOH and solidified with 0.5% Noble Agar.

- b. In a separate beaker, dissolve 40 mg acetosyringone in 5 mL 95% ethyl alcohol and add 10 mL 100X B5 vitamin stock, 0.25 μ L GA₃ stock, and 1.67 mL BAP stock per liter of basic medium and filter-sterilize.
 - c. Prepare three separate solutions of 1000 mg/L L-cysteine (final concentration 8.8 mM), 158 mg/L sodium thiolsulfate (1 mM), and 154 mg/L dithiothreitol (1 mM). Each thiol compound is dissolved in 5 mL water per liter and kept separate from one another during preparation to reduce the sulfur odor (see **Note 1**).
 - d. Add acetosyringone solution and thiol compound solutions to cooled SCCM and pour into 15 $\times$ 100-mm Petri dishes.
 - e. After medium solidifies, place a single sterile Whatman #1 (70-mm) filter paper (Whatman International, Maidstone, England) on the medium in each plate.
 3. Shoot induction medium (SIM):
 - a. Autoclave full-strength B5 major and minor salts, MSIII iron, 3% sucrose, 0.59 g/L MES, adjusted to pH 5.6 with 10 N KOH and solidified with 0.8% Noble Agar (Difco).
 - b. Mix together 10 mL 100X B5 vitamins, 1.67 mL BAP, 500 mg ticarcillin (TICAR), 100 mg cefotaxime (Claforan).
 - c. Filter-sterilize mixture with a 25-mm syringe filter with a 0.2- μ m size pore Acrodisc filter.
 - d. Add hygromycin B when appropriate.
 - e. Add the sterilized mixture to the cooled medium and pour into 25 $\times$ 100-mm Petri dishes.
 4. Shoot elongation medium (SEM):
 - a. Autoclave full-strength MS major and minor salts, MSIII iron, 3% sucrose, 0.59 g/L MES, adjusted to pH 5.6 with 10 N KOH and solidified with 0.8% Noble Agar (Difco).
 - b. Mix together 10 mL 100X B5 vitamins, 50 mg L-asparagine, 100 mg L-pyrogutamic acid, 0.1 mL IAA, 0.5 mL GA₃, 1 mL ZR, 500 mg ticarcillin, and 100 mg cefotaxime.
 - c. Filter-sterilize mixture using a 25-mm syringe filter with a 0.2- μ m filter.
 - d. Add the mixture to the cooled medium and pour into 25 $\times$ 100-mm Petri dishes.
 5. Rooting medium (RM):
 - a. Autoclave 1/2-strength B5 major and minor salts, full-strength MSIII iron, 2% sucrose, 0.59 g/L MES, adjusted to pH 5.6 with 1 N KOH and solidified with 0.8% Noble Agar (Difco).
 - b. Add 1 mL IBA stock to the cooled medium and pour into sterile vials.

2.6. Other Supplies and Materials

1. Number 15 Personna Plus surgeon's blade (American Safety Razor Company, Staunton, VA).

2. For greenhouse supplies, bags of perlite and peat moss from any manufacturer, pasteurized soil (sandy loam preferred), and 2.5-gallon pots or larger. A slow-release fertilizer (15-9-12) or liquid fertilizer (21-5-12) can be used when needed.

3. Methods

3.1. Seed Sterilization and Seedling Growth

1. Chorine gas seed sterilization:
 - a. Place two layers of seeds in a 15×100 -mm plastic Petri dish.
 - b. In an exhaust fume hood, place seed into a glass desiccator with a 250-mL beaker containing 100 mL bleach (5% NaOCl) and slowly add 3.5 mL 12 N HCl to the beaker.
 - c. Seal the lid on the desiccator and sterilize the seeds for at least 24 h. For extremely contaminated seeds, repeat this procedure.
 - d. Seeds can be stored in sealed Petri plates until use.
2. Germinate approx 16 seedlings per Petri plate containing GM at 25°C under 18-h light/6-h dark cycle at 90 to 150 $\mu\text{E}/\text{m}^2/\text{s}$ and grow until the cotyledons turn green but before the first true leaves grow completely out of the cotyledon (see **Note 2**). The seedlings can be used immediately for transformation or stored at 4°C overnight or until the *A. tumefaciens* is ready for inoculation. We have found that seedlings are susceptible for transformation even after 5 d at 4°C.

3.2. *A. tumefaciens* Preparation

1. Streak *A. tumefaciens* from a permanent glycerol stock onto solid YEP growth medium and incubate for about 2 d at 25°C or until colonies appear. Depending on the selectable marker genes present on the Ti plasmid, the binary vector, and the bacterial chromosomes, different selectable agents will be used for *A. tumefaciens* selection in the YEP solid and liquid media. For binary vectors containing 35Sp-*hpt* without an intron, 50 mg/L hygromycin B can be used for *A. tumefaciens* selection.
2. Pick a single colony with a sterile toothpick, inoculate into 50 mL of liquid YEP medium with antibiotics, and incubate (175 rpm) at 25°C for approximately 2 d. After an OD_{600} between 0.8 and 1.0 is reached, make a 15% glycerol stock with the broth, make 1-mL aliquots in 1.5-mL Eppendorf tubes, and store at -80°C (see **Note 3**).
3. One day before explant inoculation, add two to three Eppendorf tubes of *A. tumefaciens* glycerol stocks plus appropriate antibiotics (200 μL hygromycin B stock) to 200 mL YEP liquid medium in a 500-mL Erlenmeyer flask. Incubate the flask overnight at 25°C (125 rpm) until an OD_{600} between 0.8 and 1.0 is reached (see **Note 4**).
4. Before preparing and inoculating soybean explants, divide the broth into 50-mL aliquots and pellet *A. tumefaciens* by centrifugation for 10 min at 3270g at 20°C. Resuspend the pellet in 25 mL liquid CCM and place at room temperature at least 30 min before use. This makes the final OD_{600} approximately 2.0.

3.3. Explant Preparation and Inoculation

1. After the *A. tumefaciens* is resuspended in 25 mL liquid CCM, begin preparing the soybean cotyledonary nodes.
2. Excise the root and the majority of the hypocotyl approximately 3 to 5 mm below the cotyledonary node using a number 15 Personna Plus surgeon's blade. Bisect the cotyledons by cutting vertically through the hypocotyl region to produce two equal halves, each containing a cotyledon, half of the hypocotyl, and half of the epicotyl tissue.
3. Remove the epicotyl at the base of the cotyledonary node by a single cut. In addition, remove all preformed axillary shoots (easily identified as pubescent vegetative growth), taking care to avoid damaging meristem cells located within this tissue. Removing the epicotyl and preformed axillary shoots suppresses apical dominance, thereby inducing *de novo* proliferation from the axillary meristems.
4. Finally, wound the axillary meristems and the cotyledonary node by slicing approx 10 times with the blade perpendicular to the hypocotyl. When wounding, cut deep enough to access the meristematic tissue but avoid removing or extensively damaging the tissue (see **Note 5**). **Figure 1A** shows an example of an explant ready for *A. tumefaciens* inoculation.
5. About 50 explants are transferred to a Petri dish containing 25 mL of CCM/*A. tumefaciens* suspension for approximately 30 min. Explants can be moved to the suspension either after each wounding or after a group of explants are wounded. (We cut approx 50 before moving.)
6. After inoculation, five explants are plated adaxial or wounded side down on the Whatman paper overlaying SCCM (**Fig. 1B**). Filter papers prevent *A. tumefaciens* overgrowth on the soybean explants. Wrap five plates with Parafilm "M" (American National Can, Chicago, IL) and incubate for 5 d in the dark at 25°C (see **Note 6**).

3.4. Selection and Plant Regeneration

1. Excess *A. tumefaciens* is removed by briefly immersing the explants into liquid SIM (see **Note 7**).
2. Rinsed explants (five per plate) are perpendicularly imbedded hypocotyl end down into solid SIM not containing hygromycin B so that the hypocotyl and wounded cotyledonary-node tissue are under the medium surface (see **Note 8**). Wrap plates with Scotch 394 venting tape (3M, St. Paul, MN) and place in a growth chamber for 14 d at 25°C under an 18-h light/6-h dark cycle at 90 to 150 $\mu\text{E}/\text{m}^2/\text{s}$ (see **Note 9**).
3. Transfer explants to SIM containing 5 mg/L hygromycin B after carefully excising the hypocotyl. Also remove very long shoots since they arose from preformed shoots that were not removed during explant preparation. Incubate in the growth chamber for an additional 14 d.
4. After 28 d on SIM (**Fig. 1C**), remove the cotyledon and any dead tissue and transfer explants to SEM containing 10 mg/L hygromycin B. Incubate in growth chamber for an additional 14 d (see **Notes 10 and 11**).

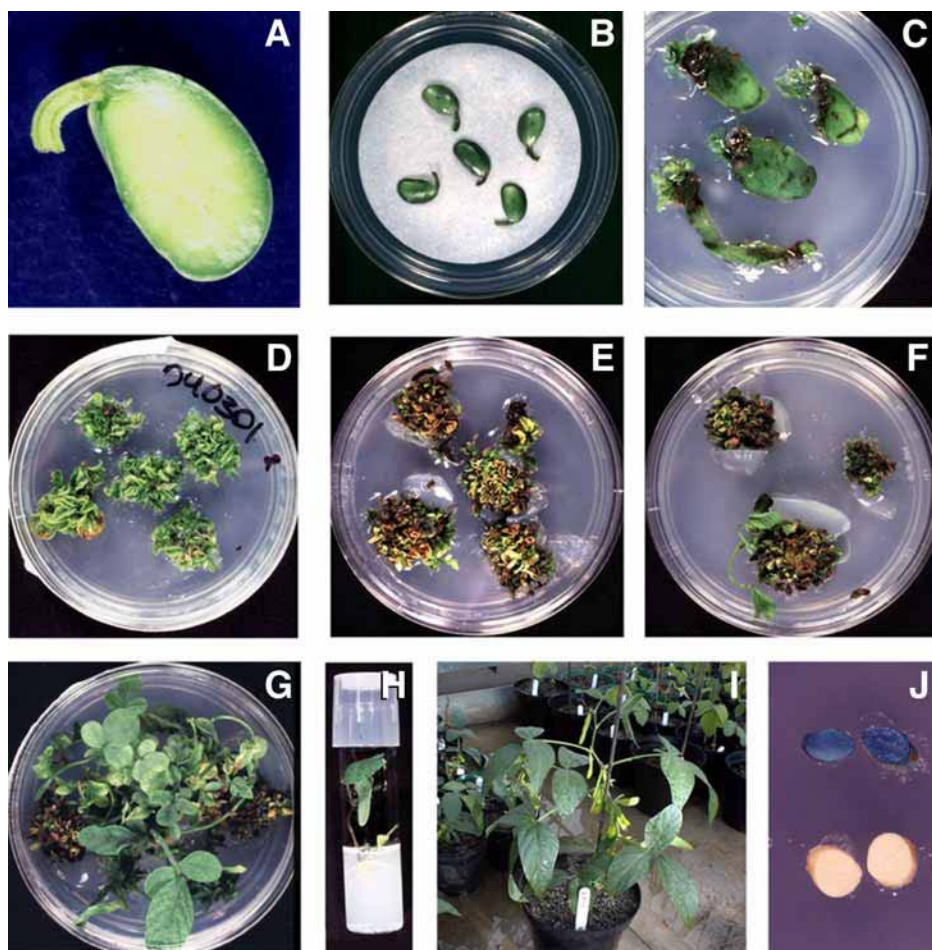


Fig. 1. The cotyledonary-node transformation method using hygromycin B selection. **(A)** Explants prepared from 5-d-old seedlings by removing the roots and the majority of the hypocotyl and wounding the axillary meristematic tissue at the cotyledonary-node (bar = 0.4 cm). **(B)** Inoculated explants cocultivated with *A. tumefaciens* on SCCM containing thiol compounds (bar = 2.5 cm). **(C)** After 5 d, the cotyledonary node and hypocotyl of the explants are embedded into solid shoot induction medium (SIM) to stimulate *de novo* shoot formation from the wounded axillary meristematic tissue (bar = 1.8 cm). **(D)** Explants cultured on SIM without hygromycin for 14 d followed by culture on SIM containing 5 mg/L hygromycin B for 14 d (bar = 2.5 cm). **(E)** Significant death of nontransformed shoots and callus tissue occurs after culturing explants for 28 d in shoot elongation medium (SEM) containing 10 mg/L hygromycin B (bar = 2.5 cm). **(F,G)** Two months after cocultivation, explants were maintained on SEM containing up to 10 mg/L hygromycin B. Examples of transformed shoot formation on explants at various times: shoot elongation on an explant 3 mo after cocultivation

5. After every 2 to 3 wk, transfer explants to fresh SEM medium after excising dead tissue. The explants should hold together and not fragment into pieces and should retain somewhat healthy sectors up until 8 wk after cocultivation (4 wk on SEM; **Fig. 1D** and **E**). After this time, it is normal that many shoots are lost and some explants turn a caramel brown color; however, there should also be some explants with healthy vigorous shoot elongation at this time, as seen in **Fig. 1F** and **G** (see **Note 12**).
6. To increase shoot production from a promising explant, push the developing shoot cluster into the medium to induce further axillary shoot growth from the nodes. In several weeks, most of these shoot clusters will develop into proliferating shoot cultures that are clones of one another.
7. Healthy, vigorous shoots with at least three sets of leaves that are preferably over 4 cm in length are excised and plated on RM (**Fig. 1H**). In a typical experiment at least 90% of the shoots form roots at the cut site after 5 to 14 d on RM. It is common that roots also form while the shoots are still in SEM. When this happens, transfer to RM for several days before transferring to the greenhouse.

3.5. Transplantation and Greenhouse Maturation

1. Once a small root is formed on the shoot, the plantlets are transferred directly to large pots (2.5 gallons or larger) filled with a mix of $\frac{1}{2}$ pasteurized top soil (sandy loam): $\frac{1}{4}$ perlite: $\frac{1}{4}$ peat moss in the greenhouse. We have found that a hardening-off period in a smaller pot is not necessary in our hands, and it is not worth the time it takes to do two transfers. The greenhouse conditions are set to 28°C with a 16/8-h (light/dark) photoperiod (see **Note 9**) under natural lights supplemented with 1000-W high-pressure sodium lamps. We try to provide a minimum of 200 $\mu\text{mol}/\text{m}^2/\text{s}$ of light.
2. To reduce moisture loss and to protect the shoot from the surrounding environment, cover the plantlets with a clear plastic container and remove when the first set of new leaves opens. When environmental conditions are optimal, greater than 90% of the rooted shoots develop into healthy, fertile plants.
3. The plants are fertilized with slow-release pellets containing 15-9-12 (N-P-K) or with a liquid fertilizer containing 21-5-12 when plants show nutrient deficiencies (usually when the plants begin yellowing).
4. The seed-set for T_0 soybean plants is highly variable and, when plants are grown in a greenhouse, is sensitive to the time of year (photoperiod and light intensity),

(figure caption continued from previous page) (**F**; bar = 2.5 cm) and shoot proliferation 4 mo after cocultivation (**G**; bar = 2.5 cm). (**H,I**) Shoots elongated to at least 4 cm in length are placed in rooting medium (**H**; bar = 2.5 cm) and rooted shoots are directly transferred to a greenhouse and grown to maturity (**I**). (**J**) A sample of T_1 seeds from each T_0 plant was stained for GUS expression; an example of a cross-section of a GUS⁺ seed (left) alongside a GUS⁻ seed (right) is shown (bar = 1 cm). (Reprinted with permission from **ref. 15**.)

cultivar, maturity zone of greenhouse, and environmental pressures (especially pests). Plants may produce as little as one seed to hundreds of seeds, so one should take into consideration what photoperiod is optimal for each environment and use supplemental lighting or shading when needed. Using this method, the typical maturation period is approximately 3 to 4 mo.

4. Notes

1. Addition of all three thiol compounds in the SCCM resulted in the highest transformation efficiency over the addition of only one or two of the thiol compounds using the cotyledonary-node explant method (15). For other soybean genotypes and explant tissues, both concentrations and combinations of thiol compounds in the SCCM may need to be empirically determined to achieve optimal transformation efficiencies.
2. The actual length of time on GM is dependent on numerous factors including genotype, health of the seed, and light intensity. Normally it takes anywhere from 3 d for small-seeded cultivars to 5 to 7 d for large-seeded cultivars. A good indicator of optimal explant development, is when the first true leaves are 4 to 10 mm in length.
3. Unless a minimal nutrient medium is required (e.g., AB medium) for bacterial growth, YEP is used for both solid and liquid medium.
4. It is common for some *A. tumefaciens* strains like LBA4404 to grow in clumps in the liquid medium. This will not affect transformation. Occasional mixing or a quick vortexing of the *Agrobacterium*, including during cocultivation, can be done to break up the clumps.
5. It is best to dissect the soybean explants under magnification. The target cells are located in the tissue between the hypocotyl and the cotyledon, which can be hard to reach with the scalpel. However, on a good explant, one can see circular tissue growth around the target tissue.
6. We find that infection is greater after 4 or 5 d of incubation rather than 1 to 3 d. *A. tumefaciens* overgrowth is not a problem with long incubations using this explant tissue.
7. At this time in the protocol it is helpful to sample the explants for transient GUS expression if the *gusA* gene was on the binary vector used for transformation. Although not necessarily an accurate indicator of transformation efficiency, this test helps to determine whether there were any overall problems with transformation (13,14). In a good transformation experiment, 80 to 100% of explants will have transient GUS expression on the hypocotyls, cotyledonary-node region, and/or cotyledons.
8. Explants at this stage in development are too sensitive to hygromycin B and will die if exposed to even low levels. Therefore, the concentration of hygromycin B is gradually increased to reduce initial cell death throughout shoot induction to shoot elongation. For this protocol, the concentrations reported are for the soybean cultivar 'Bert'. If using other cultivars, the concentration of hygromycin B may need to be increased or decreased throughout shoot initiation and elonga-

tion. Nevertheless, for the first 2 wk on SIM, no selection is recommended for all cultivars. If weaker selective agents are used, it may be necessary to expose the explants to selection immediately after cocultivation.

9. Day length is very important. Cultivars in maturity groups lower than 2 require a long day length to induce vegetative growth and prevent flowering.
10. The frequency of regeneration can be calculated by: [(number of explants with shoots/total number of explants inoculated) $\times$ 100]. We find that a good transformation experiment with the Bert genotype gives us 80 to 100% regeneration of shoots. If the frequency is lower, there are usually problems with *A. tumefaciens* overgrowth or poor transformation. The regeneration rate is also cultivar dependent; therefore, one should test the regeneration capacity of a prospective cultivar before beginning a transformation experiment.
11. It is helpful to sample the explants for stable GUS expression after 4 wk on shoot induction if the *gusA* gene was on the binary vector used for transformation (13,14). We routinely sample explants from transformation experiments and look for GUS-expressing shoots and callus tissue. In a typical experiment approximately 20% of the explants will have large sectors of transformed shoots with significant GUS staining in the callus tissue.
12. Using thiol compounds in the cocultivation medium leads to an increase in transgenic cells in the explants, especially on any surface that was initially cut before inoculation. When you are using other methods for selection, such as PPT or glufosinate selection, it may be necessary to increase the concentration of selective agent used over explants not treated with thiol compounds during cocultivation to kill the nontransgenic cells in the callus/shoot mass effectively (17).

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Soybean (*Glycine max*) Transformation Using Immature Cotyledon Explants

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Summary

Agrobacterium tumefaciens-mediated transformation of soybeans can be accomplished using immature zygotic cotyledons as target tissues providing an alternate explant to embryogenic tissue cultures, proliferating meristems, and cotyledonary nodes. The immature cotyledon method includes direct induction of transgenic somatic embryos from the explant plated on selective media after cocultivation, followed by maturation and regeneration of individual somatic embryos into whole plants. Although this method has been improved to be simple, rapid, reproducible, and applicable to a range of cultivars in different maturity groups, the transformation efficiency (Southern-positive, independent plants produced per 100 immature cotyledon explants) is 1.7% and needs to be further increased to make this a robust soybean transformation system. Further refinements of cocultivation conditions, tissue culture, and selection of regenerated transgenic plants will probably result in increases in transformation efficiency.

Key Words: *Agrobacterium tumefaciens*; *Glycine max*; immature zygotic cotyledon; KYRT1; genetic transformation; somatic embryogenesis.

1. Introduction

Various target tissues including shoot meristems (1), proliferative embryogenic cultures (2), and cotyledonary node explants (3) are routinely used for soybean transformation. However, until recently immature cotyledons have not been considered ideal target tissues for transformation, mainly because of their low frequency of primary somatic embryo induction and observed chimerism (4–6). The first report of fertile transgenic plants recovered from *Agrobacterium*-mediated transformation using immature cotyledons as target tissues was by Yan et al. (7). This transformation system was constrained mainly by inefficient induction of transgenic somatic embryos following

cocultivation with *A. tumefaciens* and culturing in the presence of selective agents. Recently, these limitations were overcome by use of an appropriate *A. tumefaciens* strain, coupled with the choice of explant orientation on selective medium (8–10). Production of transgenic somatic embryos was increased when immature cotyledons were cocultivated with the binary strain KYRT1 carrying the partially disarmed virulence helper plasmid and then incubated with the adaxial side facing the selection medium. This improved transformation system is relatively rapid, requiring 4 to 5 mo to produce transgenic plants from a range of cultivars that are phenotypically normal and fertile (8,11). It minimizes tissue culture-induced genetic variation because long-term liquid propagation of somatic embryogenic cultures is not required. Given that cotyledon-stage somatic embryos are similar in development and composition to developing seeds, the immature cotyledon transformation system has the additional advantage of allowing functional genomics investigations of seed development for modification of soybean seed composition. This chapter provides researchers with a new route for soybean transformation utilizing immature cotyledons as target tissues.

2. Materials

2.1. *A. tumefaciens* Strains

A. tumefaciens strain KYRT1 (12; see **Note 1**), transformed with a binary plasmid pCAMBIA1305.1 (8), is used in this chapter. This plasmid contains chimeric genes for hygromycin phosphotransferase (HPT) and β -glucuronidase (GUS) with an intron to detect plant-specific GUS expression.

2.2. Plant Materials

Soybean [*Glycine max* (L.) Merr.] plants grown under field or greenhouse conditions are used to collect immature seeds for explant preparation. We used the cultivars ‘Jack’ and ‘Bert’ from the USDA Soybean Germplasm Collection at the University of Illinois and the Minnesota Crop Improvement Association, respectively (see **Note 2**).

2.3. Media Stock Solutions

All stocks are made with double-distilled water (ddH₂O) and stored at 4°C unless otherwise noted.

1. 10X MS major salts: 200 mM NH₄NO₃, 200 mM KNO₃, 30 mM CaCl₂, 15 mM MgSO₄, 12.5 mM KH₂PO₄.
2. 100X MS minor salts: 10 mM H₃BO₃, 13 mM MnSO₄, 3 mM ZnSO₄, 0.5 mM KI, 0.1 mM Na₂MoO₄, 0.01 mM CuSO₄, 0.01 mM CoCl₂.
3. 100X MSIII iron: 10 mM FeSO₄, 10 mM NaEDTA.

4. 100X B5 vitamins: 55 mM myoinositol, 0.8 mM nicotinic acid, 0.5 mM pyridoxine-HCl, 3 mM thiamine-HCl.
5. 2,4-Dichlorophenoxyacetic acid (2,4-D; Sigma): 40 mg/mL stock solution. Dissolve 1 g 2,4-D in 25 mL of 95% ethyl alcohol.
6. Hygromycin B (Boehringer Mannheim): 50 mg/mL stock solution. Dissolve 1 g hygromycin B in 20 mL ddH₂O. Filter-sterilize. Store at -20°C.
7. Cefotaxime (Claforan, Aventis Pharmaceuticals): 250 mg/mL stock solution. Dissolve 2.5 g in 10 mL ddH₂O. Filter-sterilize. Store at -20°C.
8. Acetosyringone (Sigma): 20 mg/mL stock solution. Dissolve 200 mg in a 10 mL of 95% ethyl alcohol. Store at -20°C.

2.4. Culture Media for *A. tumefaciens*

The following recipes use the stock solutions given in **Subheading 2.3.** above and are calculated per liter total volume.

1. Yeast extract peptone medium (YEP): 1% Bacto-peptone (Difco), 0.5% yeast-extract (Difco), 0.5% NaCl, adjusted to pH 7.0 with 1 N NaOH, solidified with 1.5% granulated agar (Difco) and poured into 15 × 100-mm Petri dishes.
2. Liquid cocultivation medium (LCCM): full-strength MS major and minor salts, B5 vitamins, MSIII iron, 1 mL 2,4-D stock, and 3% sucrose adjusted to pH 7.0. Add acetosyringone to final concentration of 40 mg/L to cooled autoclaved LCCM.

2.5. Soybean Tissue Culture Media

1. Solid cocultivation medium (SCCM): full-strength MS major and minor salts, MSIII iron, B5 vitamins, 1 mL 2,4-D stock, 3% sucrose adjusted to pH 7.0 and solidified with 0.8% Noble Agar (Difco). Add acetosyringone to final concentration of 40 mg/L to cooled autoclaved medium. Pour into 25 × 100-mm Petri dishes.
2. Somatic embryo (SE) induction medium I (SEIM I): full-strength MS major and minor salts, MSIII iron, 1 mL 2,4-D stock, 3% sucrose adjusted to pH 7.0 and solidified with 0.2% Gelrite gellan gum (Sigma). Add 500 mg/L cefotaxime and 10 mg/L hygromycin stock to the cooled medium and pour into 25 × 100-mm Petri dishes.
3. SE induction medium II (SEIM II): SEIM I plus 25 mg/L hygromycin.
4. SE induction medium III (SEIM III): SEIM II plus 20 mg/L 2,4-D concentration; adjust pH to 5.8.
5. Maturation medium (MM): full-strength MS major and minor salts, MSIII iron, 6% maltose, adjusted to pH 5.8 and solidified with 0.8% Noble Agar. Add 10 mg/L hygromycin and 500 mg/L cefotaxime to the cooled medium and pour into 25 × 100-mm Petri dishes.
6. Regeneration medium (RM): full-strength MS major and minor salts, MSIII iron, 3% sucrose, adjusted to pH 5.6 with 0.2 N NaOH. Add 2 g Gelrite gellan gum, autoclave, and pour into 25 × 100-mm Petri dishes or Magenta GA-7 boxes (Magenta, Chicago, IL).

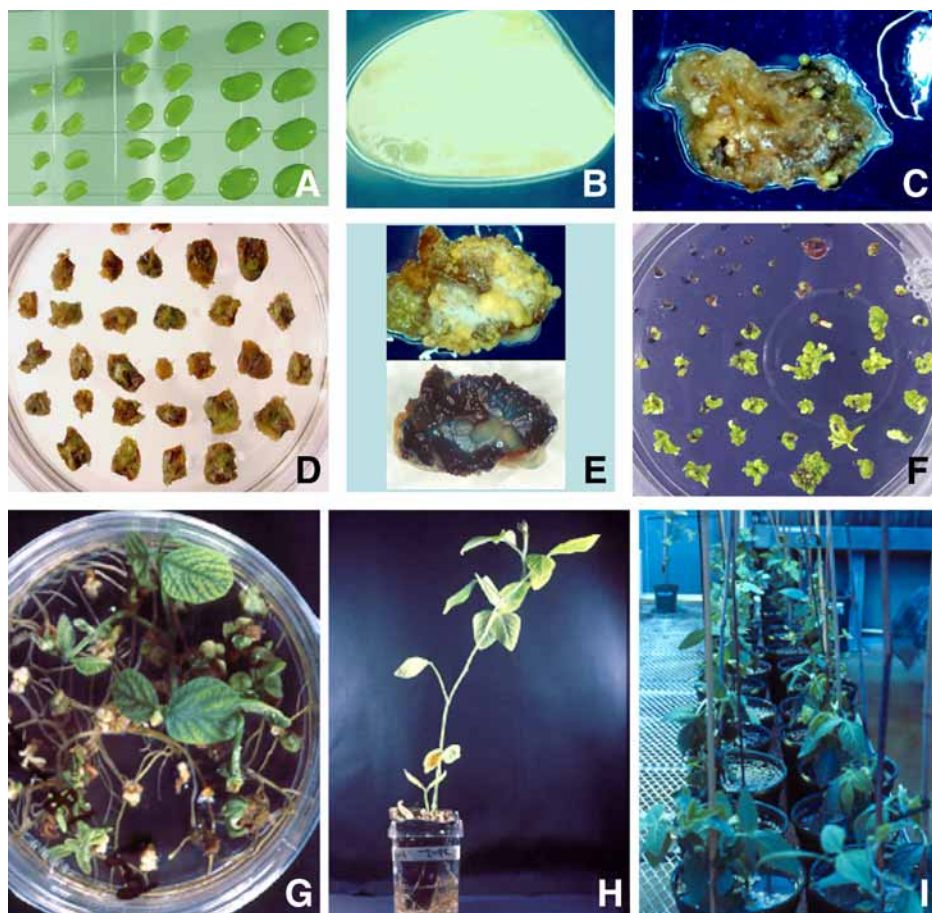


Fig. 1. *Agrobacterium*-mediated transformation of immature cotyledonary explants, somatic embryo induction under hygromycin selection, and recovery of transgenic plants. **(A)** Immature embryos of 5 to 6 mm (left), 7 to 8 mm (middle), and 9 to 10 mm (right) in length removed from sterilized pods. **(B)** Explants prepared from immature embryos (5–8 mm in length) by removing the embryonic axis and squeezing it out of the seed coat. After 4 d of cocultivation with *A. tumefaciens* on SCCM, explants (adaxial side down) are incubated on SEIM I for 2 wk and then SEIM II for 2 wk to induce transgenic somatic embryos. **(C)** Green globular somatic embryos emerging along the margins of an explant, indicated by arrows. **(D)** To maximize production of transgenic somatic embryos, explants are subcultured biweekly on SEIM III until somatic embryos are no longer induced. At 8 to 10 wk after cocultivation, a highly embryogenic explant can be identified by browning/necrotic tissues along the margins and collapsed cells in the middle area. **(E)** A highly embryogenic explant producing a mass of hygromycin-resistant somatic embryos (top) and stained for GUS expression (bottom) 4 wk after incubation on SEIM III. **(F)** Individual green somatic embryos are

3. Methods

3.1. Young Pod Collection and Sterilization

1. Young pods containing immature embryos (5–8 mm in length) are collected from plants grown under field or greenhouse conditions (see **Note 3**; **Fig. 1**). The pods can be used immediately for transformation or stored at 4°C up to 10 d.
2. Young pods are surface-sterilized by soaking in 70% 2-propanol for 30 s and then in a 25% (v/v) Chlorox (0.105% sodium hypochlorite) commercial bleach with vigorous shaking for 20 min. The pods are then rinsed three times with sterilized ddH₂O.

3.2. *A. tumefaciens* Preparation

1. Streak *Agrobacterium* culture from a permanent glycerol stock onto solid YEP medium (50 mg/L rifampicin and 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 5 mL of liquid YEP medium with 100 mg/L kanamycin and grown overnight at 28°C; shake at 175 rpm. Then 500 µL of overnight culture are recultured in 50 mL of liquid YEB medium (100 mg/L kanamycin) until an OD₆₀₀ between 1.3 and 1.5 is reached.
3. The bacterial culture is centrifuged at 3270g for 10 min at 20°C. The resulting pellet is washed with LCCM. Following a second centrifugation, the pellet is resuspended in 50 mL LCCM supplemented with acetosyringone (40 mg/L) and divided into 25-mL aliquots.

3.3. Explant Preparation and Inoculation

1. Immature seeds are aseptically removed from sterilized pods. Cut off and remove the end of the immature seed containing the embryonic axis. Squeeze out and separate the two cotyledons from the seed coat (**Fig. 1B**).
2. After isolation, about 200 explants are transferred to a Petri dish containing 25 mL of CCM/*A. tumefaciens* suspension for approximately 60 min.
3. Then inoculated explants are briefly blotted onto sterilized filter paper and placed on SCCM. About 50 explants are plated adaxial side down on SCCM (see **Note 4**). Wrap three plates with Parafilm “M” (American National Can, Chicago, IL) and incubate for 4 d in the dark at 25°C (see **Note 5**).

(figure caption continued from previous page) carefully removed from explants under the dissecting microscope. Hygromycin-resistant mature embryos 8 wk after culture on MM. **(G)** All mature embryos are subjected to a desiccation treatment, germination on RM, and conversion into plantlets having both shoots and roots. **(H)** A regenerated plant transferred to a small pot in a Magenta box for acclimatization. **(I)** Primary transgenic plants transferred to a greenhouse for flowering and seed set.

3.4. Selection and Plant Regeneration

1. After cocultivation, explants are washed three times with sterile ddH₂O to remove excess *A. tumefaciens* and rinsed with liquid CCM containing 500 mg/L cefotaxime.
2. Rinsed explants (25 per plate) are placed abaxial side up on SEIM I supplemented with 10 mg/L hygromycin B. Wrap plates with Parafilm "M" and incubate in a growth chamber for 14 d at 25°C under a 23-h light/1-h dark cycle provided by cool-white fluorescent tubes (10–20 $\mu\text{E}/\text{m}^2/\text{s}$).
3. Transfer explants to SEIM II containing 25 mg/L hygromycin B. Incubate in the growth chamber for an additional 14 d. After 28 d on SEIM I, explants are evaluated for somatic embryogenesis using the dissecting microscope. Early induced hygromycin-resistant somatic embryos (SEs) are usually observed on responding explants at this time (see **Note 6**).
4. To regenerate whole plants, induced SEs are carefully excised from responding explants under the dissecting microscope and directly placed on solid MM containing 10 mg/L hygromycin B. Incubate in the growth chamber for 8 wk with 2-wk subculture intervals for maturation. Maintain the temperature and light intensity as above.
5. Explants, both responding and nonresponding to 2,4-D treatment, are continuously subcultured biweekly on SEIM III containing 25 mg/L hygromycin B. More SEs are induced on previously either responding or nonresponding explants up to 8 wk after cocultivation (see **Note 7**).
6. After 8 wk of maturation, the resulting cotyledonary-stage embryos are ready for desiccation (see **Note 8**; **Fig. 1F**). When the embryos reach physiological maturity, they lose their green color and acquire a creamy yellow color. Mature SEs are then desiccated in empty Petri dishes (100 $\times$ 15 mm) at 25°C for 5 to 7 d under a 23-h photoperiod and a light intensity of 60 to 80 $\mu\text{E}/\text{m}^2/\text{s}$. A small piece (approximately 10 mm³) of MM is also placed in the dishes to allow gradual desiccation of embryos.
7. Desiccated mature embryos are then placed on RM containing 500 mg/L cefotaxime for 4 to 6 wk until both shoots and roots are formed (3–6 wk; **Fig. 1G**). The plantlets are transferred to Magenta GA-7 boxes containing 50 mL of RM for further growth.
8. The plantlets with healthy branched roots and an elongating shoot (5–10 cm) are transferred to sterile soil mix (1:1:1 of soil, peat, and perlite) in small pots, placed in Magenta GA-7 boxes, and covered with a plastic bag to maintain high humidity. These plants can be placed in a growth chamber at 25°C with 75% relative humidity, a 23-h photoperiod, and a light intensity of 200 $\mu\text{E}/\text{m}^2/\text{s}$.
9. After 7 d, the plastic bag is gradually opened up over a 7-d period to allow for acclimatization of plantlets (**Fig. 1H**).

3.5. Greenhouse Care of Transgenic Plants

1. Acclimatized plants are transferred to 6-inch pots containing soil mix (1:1:1 of soil/perlite/torpedo sand) in a greenhouse (**Fig. 1I**).

2. Plants are grown at 22 to 28°C under an 16-h photoperiod under natural light supplemented with 1000-W high-pressure sodium lamps.
3. Plants are watered daily and fertilized once every 2 wk with Peters (20:20:20) complete plant food.
4. It takes approximately 2 to 3 mo for flowering and seed set (approximately from a few dozens to hundreds) after transfer to the greenhouse, depending on genotypes and growth conditions (see **Note 9**).

4. Notes

1. The virulence helper plasmid pKYRT1 harbored in strain KYRT1 is not completely disarmed and contains two T-DNAs including an intact T-right (T_R) T-DNA (**12,13**). More than 70% of the transgenic plants contain only the T-DNA from the binary plasmid, whereas 30% of transgenic plants contain the T_R T-DNA from the virulence helper plasmid in addition to the T-DNA from the binary plasmid (**9**). Given that some frequency of the cotransformed plants will have the two T-DNAs likely integrated into different genomic regions and will segregate independently, normal transgenic plants free of the T_R -DNA can be obtained by evaluating progeny of the cotransformed plants (**9**). In addition to partially disarmed *Agrobacterium* strain KYRT1, a fully disarmed strain EHA105 can be used for transformation of immature cotyledons to recover fertile transgenic plants (**7**). However, the embryogenic response of inoculated explants under selection condition was reported to be low (**8,9**). Thus, the transformation technique described in this chapter depends on the use of KYRT1 as the *vir* helper strain.
2. We previously found that multiple soybean cultivars in a wide range of maturity groups (MG 00 to MG VIII) were amenable to transformation of immature cotyledons (**11**). In general, soybean cultivars in maturity group MG II to MG IV are highly recommended since those had a higher embryogenic response under hygromycin selection. However, there are certainly exceptions to the trends for maturity.
3. Explants from immature embryos 3 to 4 mm in length have commonly been used to induce somatic embryos (**14,15**). However, larger embryos of 5 to 8 mm in length (light, translucent green) resulted in the highest embryogenic response under stresses associated with *Agrobacterium* infection and hygromycin selection. Explants derived from embryos > 9 mm in length (darker, more opaque) showed the least embryogenic response (**Fig. 1A**). Since soybean genotypes vary widely in seed size, one should evaluate the embryogenic response of a prospective cultivar empirically.
4. Explants should be incubated with the adaxial side facing down on CCM and selective SEIM. Explant orientation is critical for production of transgenic SEs (somatic embryos) at high frequency. It is well known that when the adaxial side of immature cotyledons is oriented up on a nonselective medium, high SE induction is exhibited (**6**). However, we found that the opposite orientation of explants in the presence of *Agrobacterium* and selective agents shows high embryogenic and transformation potentials (**8,10**).

5. Cocultivation periods of 2 or 3 d have been used for transformation of soybean to avoid problems of overgrowth of *Agrobacterium* (7,16). However, we find that a high *Agrobacterium* suspension concentration and a prolonged cocultivation period significantly increase transgenic SE productions. No bacterial overgrowth was observed on cultured explants infected with a high concentration of bacterial cells.
6. Greenish yellow to green hygromycin-resistant SEs can be observed as either single/polyembryogenic globular structure or clusters of globular SEs (Fig. 1C). The majority of induced SEs on selection medium arise along the margins of explants. Not all SEs induced under hygromycin selection are transgenic. More than 50% of these SEs are transgenic based on histochemical GUS assay (Fig. 1D).
7. After the 8-wk selection period, it is possible to distinguish between highly and poorly embryogenic genotypes by observing the phenotype of cultured explants. Highly embryogenic cultivars commonly display dark brown necrotic tissues along the margins of the abaxial side of responding explants, where conditions are probably optimal owing to accessibility to 2,4-D and selective agents (Fig. 1C and E).
8. The level of hygromycin concentration used in induction media does not effectively suppress the emergence of nontransformed SEs. However, any escapes can be effectively eliminated later during maturation periods (8 wk) by continuous exposure to 10 mg/L hygromycin.
9. We find that transgenic plants recovered from previous transformation experiments with the Jack genotype show 100% fertility and appear to be normal.

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Tepary Bean (*Phaseolus acutifolius*)

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Summary

Phaseolus beans are among the major legumes for food consumption, especially in Latin America, Africa, and Asia. Tepary bean (*Phaseolus acutifolius* L. Gray) is one of the five cultivated species of the genus *Phaseolus*. This chapter describes an *Agrobacterium*-mediated transformation protocol for *P. acutifolius* based on cocultivation of callus, derived from cotyledonary nodes, with *Agrobacterium*. The selectable marker gene used is neomycin phosphotransferase II (*nptII*), and the selection agent is geneticin. Selection of transgenic callus material is achieved through four to five passages on geneticin-containing medium, after which shoots are induced on medium without selection agent. The protocol as described here has been applied to transform a cultivated variety of *P. acutifolius*, TB1, and also with some modifications to a wild genotype, NI576 and another cultivated variety, PI440795.

Key Words: *Phaseolus acutifolius*; Tepary bean; food legume; gene transfer; biotechnology; *Agrobacterium tumefaciens*.

1. Introduction

Phaseolus beans are among the most important grain legumes for direct food use (1). Beans are grown all over the world, but mainly in South and Central America, Africa, India, and China. The annual production is around 24 million tons (2). Owing to the high variability within the *Phaseolus* genus, genetic improvement by classical breeding has been quite successful (3). For most crops, the main target is to breed varieties with a high yield and improved tolerance or resistance to diseases, pests, and drought. For beans, additional objectives in classical breeding are good taste, low flatulence factors, plant architecture, and pod shattering and pod distribution that allow for easy, preferably mechanical, harvesting.

Transformation through *Agrobacterium* and regeneration of transformed cells are still troublesome in *Phaseolus* spp. (for review, see refs. 2 and 4). Transformation efficiency and regeneration ability seem to depend on genotype, explant, and tissue culture conditions. So far, reproducible *Agrobacterium*-based transformation protocols are only available for *P. acutifolius* and not for the other *Phaseolus* species, including the economically most important species, *P. vulgaris*.

The protocol described here is based on the induction of green nodular callus on explants derived from germinated seeds (5) and cocultivation of this callus with *Agrobacterium*. After cocultivation, transgenic calli are selected through four or five passages on a geneticin-containing callus induction and maintenance medium (CIM). This rather lengthy callus phase ensures that uniformly transgenic callus is obtained, that the selective agent can be omitted in the subsequent plant regeneration steps, and that essentially all obtained plants are truly transgenic (6). Starting from 50 beans, about 30 g of green, nodular callus is obtained, which in our hands gives 5 to 10 independent transformants. All transgenic plants obtained with this procedure are fertile and appear to be morphologically normal. Stable integration of the transgenes and transmission to the progeny have been demonstrated (6).

2. Materials

2.1. *Agrobacterium tumefaciens*

1. *Agrobacterium* strains: *A. tumefaciens* C58C1 Rif^R (pMP90) is used, which harbors the binary plasmids pATARC3-B1b or pATARC3-B52b (7). In addition to a gene of interest, these binary vectors contain the selection gene neomycin phosphotransferase II (*nptII*) under control of the nopaline synthase promoter and the *uidA*-intron gene under control of the cauliflower mosaic virus 35S promoter.
2. *Agrobacterium* medium (YEB) medium: 5 g/L tryptone, 1 g/L yeast extract, 5 g/L nutrient broth, 5 g/L sucrose, 0.49 g/L MgSO₄·7H₂O.
 - a. Dissolve ingredients in water.
 - b. Adjust the pH to 7.2 and bring the volume to 1 L.
 - c. For YEB plates, add solution to 15 g/L agar.
 - d. Autoclave.
 - e. When media cools to 60°C or below, add any antibiotics required for plasmid maintenance or selection.
 - f. For pATARC constructs, the YEB plates are supplemented with 100 mg/L rifampicin, 300 mg/L streptomycin, 100 mg/L spectinomycin, and 10 mg/L gentamycin.

2.2. Plant Material

A cultivated genotype of *P. acutifolius*, TB1 (8) is obtained from CIAT (Cali, Colombia).

Table 1
Composition of the Media

Compound	GM1	GM2	CIM	CCM	CWM	SIM	SDM
MS	+	+	+	+	+	+	+
Sucrose (g/L)	30	30	20	20	20	20	20
Glucose (g/L)	—	—	—	1.98	1.98	—	—
Agar (g/L)	6	6	8	—	—	8	8
Phytigel (g/L)	—	—	3 ^a	—	—	—	—
MES (g/L)	—	—	—	3.9	3.9	—	—
Acetosyringone (μM)	—	—	—	200	—	—	—
IAA (mg/L)	—	—	0.05	0.05	0.05	—	—
TDZ (mg/L)	—	—	0.10	0.10	0.10	—	—
BAP (mg/L)	1.13	—	—	—	—	1	0.10
Geneticin (mg/L)	—	—	^b	—	—	—	—
Carbenicillin (g/L)	—	—	^c	—	0.05	0.05	0.05
Coconut water (%)	—	—	—	—	—	10	10
pH	5.7	5.7	5.7	5.5	5.5	5.7	5.7

MS, Murashige and Skoog salts and vitamins (9); MES, 2-(*N*-morpholino)ethanesulfonic acid; IAA, indole-3-acetic acid; TDZ, thidiazuron; BAP, 6-benzylaminopurine.

^aSee Note 2.

^bSee Note 3.

^cSee Note 4.

2.3. Plant Media

All chemicals are from Sigma-Aldrich (St. Louis, MO). Agar is from Difco (Detroit, MI), except for germination medium (GM; Gibco-BRL, Gaithersburg, MD).

All media, listed in Table 1, are adjusted to the correct pH before autoclaving (120 kg/cm² for 20 min); all hormones (except TDZ), acetosyringone, vitamins, and antibiotics are added after autoclaving to cool (about 40°C) media (see Notes 1–4).

2.4. Petri Dishes and Jars

All callus induction media (CIM) are poured into Petri dishes (25 × 90 mm or 25 × 145 mm at the rate of 40 or 75 mL/plate, respectively; see Note 5). GM, shoot induction medium (SIM), and shoot development medium (SDM) are poured into glass jars (Ø8 cm with 8.5 cm height) with white transparent screw caps at a rate of 50 to 60 mL/jar. Cocultivation medium (CCM) is dispensed (200 mL/jar) in similar jars but 14.5 cm high (see Note 6).

2.5. *In Vitro and In Vivo Culture Conditions*

1. Growth chamber conditions: two growth chambers with cool white light ($35\text{--}40\ \mu\text{mol}/\text{m}^2/\text{s}$) and a photoperiod of 16-h light and 8-h dark were used to incubate all cultures (see **Note 7**). Growth chambers #1 and #2 had temperatures of $22 \pm 0.4^\circ\text{C}$ and $25 \pm 1^\circ\text{C}$ during the day, respectively, and $23 \pm 1^\circ\text{C}$ during the night. All cultures are sealed with gas-porous tape (Urgopore, Chenoves, France) and incubated in growth chamber #2, except for seed and cocultivated cultures, which are incubated in growth chamber #1.
2. Greenhouse conditions: grafted shoots and rooted shoots are grown in the greenhouse, after transplantation into 4.5-L pots containing soil (type 1 soil, fertilized with $1.25\ \text{kg}/\text{m}^3$ [N-P-K 14-16-18%]; M. Snebbout NV, Gent, Belgium). All plants are fertilized with 2 g of urea after 1 mo of transplanting and 2 g of urea after induction of flowering. Greenhouse conditions are 16-h/8-h light/dark cycle, a temperature of $25 \pm 2^\circ\text{C}$ and relative humidity of 60 to 70%.

3. Methods (see Note 8)

3.1. *Seed Germination*

1. *P. acutifolius* (variety TB1; see **Note 9**) seeds are surface-sterilized (see **Note 10**). Seeds are rinsed in 70% EtOH (100 mL) for 20 s and shaken vigorously.
2. Next they are immersed in 3% NaOCl (300 mL) with 6 drops Tween-20 for 10 min with gentle shaking. All further manipulations are done in a sterile flow-bench.
3. Seeds are washed four times with sterile water (about 500 mL in total).
4. Seeds are distributed in jars with GM1 medium (six to eight per jar). Seeds are pushed into the medium about $\frac{1}{2}$ of the seed size deep, with the hilum side up. Seeds are incubated at 22°C for 3 d in a culture room with 16-h light and 8-h dark.

3.2. *Callus Induction*

1. The seed coat is removed from the germinated seeds; cotyledons and embryo axes are gently separated, taking care not to break the embryo.
2. The embryo axes (five maximum per small round Petri dish) and cotyledons (10 per dish) with their adaxial (flat) side down, are placed on Petri dishes with CIM media (see **Note 5**). The small plates are used to limit contamination damage, if any. The embryos should be carefully embedded in the medium (especially the root tip and nodal region).
3. The plates are incubated in the dark at 25°C , covered with aluminum foil, for 1 wk, and then the plates are uncovered and incubated further in the light at 25°C for 3 more wk.
4. Contamination should be carefully checked for. In case of fungal contamination, even a little and confined, the explants on the whole plate are discarded. In case of bacterial contamination, remove the contaminated explant carefully and the remaining explants can still be used.

5. Green tissues (precursor of callus) appear on the explants at the nodal regions where the embryo axis and the cotyledons were attached. These green tissues are separated from either the cotyledons or the embryos, chopped into small pieces (9 mm²) and cultured further on fresh CIM medium for 3 wk at 22°C in a culture room with 16-h light and 8-h dark (see **Note 11**).
6. Green compact callus tissue is cleaned by removing brown necrotic tissue, chopped into small pieces, and cultured on fresh CIM medium for another 3 wk. Select the best (dark green and compact) callus for the transformation experiment.
7. **Step 6** is repeated 5 to 6 d before transformation to obtain freshly subcultured callus prior to the transformation (see **Note 12**).

3.3. *Agrobacterium* Culture Preparation

1. The required *Agrobacterium* strain is grown fresh on solid YEB plates supplemented with antibiotics according to the vector used. Incubate the plate at 28°C for 3 d.
2. The freshly grown bacteria are inoculated in 10 mL YEB, by dispensing 8 to 10 loops of bacteria with a 5-mm inoculation loop. The suspension is homogenized, and the OD is measured at 600 nm.
3. Two 50-mL tubes (Falcon) with 5 mL YEB without antibiotics are inoculated at a concentration of 0.1 OD and incubated at 28°C with shaking (150 rpm).
4. After 12 h these cultures are each inoculated in two 500-mL Erlenmeyer flasks with 100 mL YEB with antibiotics (not rifampicin), and incubated for another 12 h.
5. Bacteria are centrifuged for 10 min at 3000g. Pellets are washed once with cocultivation medium (CCM) and finally dispensed in a 50-mL Falcon tube containing 10 mL CCM for the 2X 100 mL bacterial culture of **step 4**.
6. The bacteria are incubated at 22°C for 4 to 6 h. OD is measured before cocultivation (make a 50X dilution for OD measurement). Usually the OD of this concentrated culture is between 25 and 30.

3.4. *Cocultivation and Washing*

1. During the *Agrobacterium* incubating period (22°C, 4–6 h), the callus is cleaned (brown tissues are removed) and chopped in pieces about 3 × 3 mm by using a fresh sterile scalpel blade. (Change the blade frequently.)
2. About 3 g of chopped callus is weighed in a sterile plate and transferred into 8 × 14.5-cm glass jars containing 200 mL CCM medium with *Agrobacterium* culture at an OD of 0.05. Usually 10 jars are used in one experiment.
3. The medium is mixed well with the callus, and the jars are incubated at 22°C (with light/dark cycle of 16/8 h) for 1 wk.
4. During this week the cultures are shaken every day to avoid formation of a bacterial film on the surface of the medium.
5. After 1 wk of cocultivation, combine callus from two or three jars by passing them through a sterilized stainless steel tea-net (see **Note 13**).

6. For the first wash the callus is put in a sterile glass container ($\varnothing$ 8 cm, height 8.5 cm) containing 150 mL callus wash medium (CWM) and shaken about 3 min vigorously by stirring with long forceps in 150 mL wash medium.
7. For the second and third washes, the callus is washed for 3 min in 150 mL wash medium with shaking by hand.
8. Washed callus is blotted dry on sterile tissue paper.
9. The callus is transferred to CIM (see **Note 5**), supplemented with 5 mg/L geneticin and 500 mg/L carbenicillin. Use about 3 large Petri plates (25 $\times$ 145 mm) for material from one wash, this being typically the callus from two to three jars, equaling 6 to 9 g of callus. Incubate callus plates for 3 wk. Growth conditions throughout the selection procedure are as described for Chamber #2.
10. To monitor infection efficiency, set one small test plate ($\varnothing$ 9 cm) of callus (take 20 pieces of callus from each wash) for a GUS test. This plate should *not* contain geneticin.
11. The callus on the test plate is checked after 4 d for GUS expression. At least half of the calli should contain GUS-positive sectors.

3.5. Selection and Regeneration

1. After 3 wk on CIM with 5 mg/L geneticin, green callus pieces are transferred to CIM media with a constant geneticin level of 20 mg/L but with a carbenicillin level that is progressively reduced by 100 mg/L at each passage (starting with 400 mg/L, then after 3 wk 300 mg/L, then after 3 wk 200 mg/L, and finally 100 mg/L).
2. To monitor transformation efficiency, β -glucuronidase (GUS) expression is checked on small parts of green, growing callus pieces after this last passage (15 wk after the washing step); GUS-positive calli are transferred to SIM, whereas negative callus is maintained for two more rounds on CIM medium, and GUS expression is checked again.
3. For regeneration, GUS-positive calli are transferred to SIM medium for two subculture periods (2 wk each subculture) and then transferred to jars with SDM medium for 3 wk.
4. Callus is maintained on SDM until shoots appear that can be grafted or that will form roots spontaneously. Usually shoots that develop later (three rounds on SDM) will root spontaneously with a good frequency, with about 50% of the shoots forming roots.

3.6. Grafting and Establishment of Plants in the Greenhouse

1. Shoots that are not rooting spontaneously can be grafted. The base of apical or nodal segments (approx 5 mm) of 2- to 4-cm shoots is trimmed to obtain a wedge shape.
2. Wild-type TB1 seeds are sown and germinated for 4 d on GM2 medium to obtain root stocks. The hypocotyls of the seedlings are cut about 1.5 cm above the root by a transverse cut in the epicotyl region to remove the plumule.

3. The scion base was inserted into a vertical hole, made with a needle in the epicotyl region of the root stock. The resulting grafts were cultured on MS medium and incubated in growth chamber #2 for 3 wk to become established.
4. In vitro established grafted shoots and spontaneously rooted shoots are transplanted to soil in 4.5-L pots after washing the roots thoroughly with running tap water. Initially the plants are covered with transparent plastic to maintain humidity; thereafter, the plants are gradually acclimatized by cutting holes in the plastic to lower the humidity.
5. All plants are fertilized with 2 g urea per plant after 1 mo of transplanting and 2 g urea again after induction of flowering.
6. It takes about 3 to 4 mo for the plants to flower and set seed. All plants are fertile, but yield can vary between a few tens of seeds up to 100.
7. Efficiency can be described as 1 to 2%: from 50 seeds, 30 g of callus, equaling about 500 to 700 explants (pieces of callus), can be obtained, which will give rise to 5 to 10 independent transformants as assayed by GUS staining and Southern analysis.

4. Notes

1. SIM and SDM are made up to 900 mL only before autoclaving, after which the sterilized coconut water is added (100 mL).
2. For the first passage after cocultivation, media used contained Phytigel as a solidifying agent, instead of agar.
3. Geneticin is only added for media used after transformation.
4. Carbenicillin is only added in CIM media used after transformation.
5. CIM medium is poured rather thick (1 L medium for about 20–25 plates 2.5 cm high and 9-cm-diameter Petri dishes).
6. Jars can be closed immediately after pouring, if needed in more than 1 wk; otherwise dry for 30 min in a sterile flow-bench.
7. We have observed that light is a crucial factor for efficient transformation (10).
8. For color pictures of the different tissue culture steps, see ref. 6.
9. We have also been using a wild variety, NI576 (11,12), and another cultivated variety, PI440795 (6). See the publications of our group as indicated for more details. The protocols are based on the same principle but differ substantially in the explant with which they start.
10. Usually 50 to 100 seeds are used for one transformation experiment; this will give 5 to 10 independent transformants.
11. The callus response rate may vary, but usually about half of the explants will give some callus material. Hypocotyls are generally more responsive than cotyledons.
12. The subculture times are tightly respected. We have noticed that younger callus, as well as older callus, does not have good transformation and/or regenerability.
13. The tea-net is used as a sieve. It is sterilized by flaming with alcohol. **Caution:** the net may hold a lot of alcohol, which gives a large flame.

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VI ---

VEGETABLE PLANTS

Brassica oleracea

Penny A. C. Sparrow, Philip J. Dale, and Judith A. Irwin

Summary

A better understanding of the genetic basis underlying the genotype dependence of *Brassica oleracea* transformation is enabling researchers to distinguish between recalcitrant and successful candidate genotypes for routine transformation. In this chapter we outline an *A. tumefaciens*-mediated transformation method for *B. oleracea* using 4-d-old cotyledonary explants and a model *B. oleracea* doubled haploid genotype, AG DH1012. After only 3 wk on kanamycin selection, the first transgenic shoots can be isolated. Transformation efficiencies in the region of 10 to 25% (based on 50–125 PCR-positive independent shoots from 500 infected explants) are typically achieved. For researchers wishing to use their own plant genotype, we highlight the tissue culture phenotypes that are conducive to efficient transformation.

Key Words: *Brassica*; *Agrobacterium tumefaciens*; transformation; tissue culture blackening.

1. Introduction

The production of transgenic plants as a research tool for testing gene function is now a routine procedure for many model species, such as *Arabidopsis*, tobacco, and rice. Plant transformation is enabling researchers to introduce genes with known function from the model plants into crop plants. It is also increasingly used to introduce homologous sequences from the crops, back into the crops, either to overexpress or to silence native genes. A better understanding of gene function in crops will facilitate the transfer of knowledge of plant systems from model species and support targeted selection of key traits in plant breeding programs.

Transformation of *Brassica oleracea* (e.g., cabbage, broccoli, kohlrabi, cauliflower, Brussels sprouts, and kale) using *Agrobacterium tumefaciens* has been reported by a number of groups (1–7). Despite considerable advances in



Fig. 1. Cotyledonary petioles from four separate genotypes of *B. napus* photographed in vitro after 16 d in culture. Explant (A) shows slight swelling of the petiole base and the presence of tissue culture blackening; in explant (B) early callus formation to the petiole base is observed and tissue culture blackening is absent. Shoot regeneration via (C) a direct mode associated with tissue culture blackening and (D) an indirect callus mode in the absence of tissue culture blackening.

methodologies over recent years, the routine transformation of *B. oleracea* is still hindered by genotype restrictions, with some genotypes remaining recalcitrant to transformation. For gene testing to become a routine procedure, it is important to identify easy to transform genotypes, with reproducible and reliable transformation efficiencies, that respond well when handled by different users (7,8; see Note 1 and Fig. 1).

We have identified a number of key factors affecting transformation that are highly genotype dependent; these include susceptibility to *Agrobacterium* and in vitro tissue culture response (9,10). These studies have shown a number of stages within the transformation process to be under strong genetic control, suggesting that altering the tissue culture conditions alone will have a limited effect on transformation and regeneration efficiencies of recalcitrant material. This has led to a better understanding of why some genotypes can be trans-

formed, whereas others remain recalcitrant, and has enabled the screening of germplasm to identify high-throughput candidate genotypes for routine transformation.

In this chapter we describe a method for the transformation of *B. oleracea*; this protocol can also be applied for transformation of *B. napus* (e.g., oilseed rape/canola). The accompanying notes highlight tissue culture phenotypes that are conducive to efficient transformation and those that are likely to remain recalcitrant (see **Note 1**).

2. Materials

2.1. Plant Culture Media and Components

1. MS basal medium: 4.3 g/L Murashige and Skoog (**11**) (MS) salts (Duchefa, cat. no. M0222), 30 g/L sucrose, pH 5.7, 8 g/L Phytagar (Duchefa, cat. no. P1003). Autoclave at 120°C for 20 min (see **Note 2**).
2. 6-Benzylaminopurine (BAP; Sigma, cat. no. B-9395) stock solution: 4 mg/mL. Prepare by dissolving the powder in a few drops of 1 M NaOH. Make to final volume with sterile distilled water (SDW). Store at 4°C.
3. Vitamin stocks: 10 g/L myoinositol (Sigma, cat. no. I-3011), 10 mg/L thiamine-HCl (Sigma, cat. no. T-3902), 1 mg/L pyridoxine (Sigma, cat. no. P-8666), 1 mg/L nicotinic acid (Sigma, cat. no. N-0765). All vitamins are made up in SDW, filter-sterilized and stored individually at 4°C, with the exception of myoinositol, which is stored at room temperature.
4. Kanamycin monosulfate (Sigma, cat. no. K1377) stock solution: 100 mg/mL. Prepare by dissolving 1 g of powder in 10 mL of SDW. Filter-sterilize, store in 1-mL aliquots in sterile 1.5-mL tubes, at -20°C (see **Note 3**).
5. Carbenicillin (Duchefa, cat. no. C0109) stock solution: 500 mg/mL. Filter-sterilize and store at -20°C in 1-mL aliquots.
6. Germination medium: 1 L MS basal medium, plus 1 mL of each of the four vitamin stocks added prior to pouring. One liter typically fills 30 Petri dishes (15 × 90 mm).
7. Cocultivation medium: 1 L MS basal medium plus 2 mg/L BAP (0.5 mL of 4 mg/L BAP stock solution added prior to autoclaving); prior to pouring add 1 mL each of the four vitamin stocks. One liter typically fills 30 Petri plates (15 × 90 mm).
8. Selection medium: as cocultivation medium, with the addition of 15 mg/L kanamycin (150 µL of kanamycin stock) and 500 mg/L carbenicillin (1 mL of carbenicillin stock), added prior to pouring. One litre typically fills 20 Petri plates (20 × 90 mm). Kanamycin is not included in the control plates. (A single control plate can be poured before kanamycin is added to the medium for the other plates.) See also **Subheading 3.4**.
9. Gamborg's B5 medium (**12**): 3.1 g/L Gamborg's B5 salts (Duchefa, cat. no. G0209), 10 g/L sucrose, pH 5.7, 8 g/L Phytagar (Duchefa, cat. no. P1003). Autoclave at 120°C for 20 min. Prior to pouring, add filter-sterilized 25 mg/L kanamycin (50 mg/L for later steps) and 500 mg/L carbenicillin.

10. Sterile peat: sterile peat pots (Jiffy No. 7) are placed into Magenta pots (Sigma) and soaked in water until fully expanded. Excess water is poured off, and the Magenta pots are autoclaved at 120°C for 20 min.

2.2. *Agrobacterium Culture Media*

1. Minimal A liquid medium (for 1 L): 50 mL 20X minimal A salts (20 g/L $[\text{NH}_4]_2\text{SO}_4$ and 10 g/L sodium citrate), 50 mL 20X minimal A buffer (274 g/L K_2HPO_4 and 90 g/L KH_2PO_4), 10 mL 20% sucrose, and 1 mL 1 M MgSO_4 made up to 1 L with sterile SDW. All components should be autoclaved separately before combining, with the exception of MgSO_4 , which should be filter-sterilized as it is unstable at high temperatures.
2. LB medium: 5 g/L yeast extract (Duchefa, cat. no. Y1333), 10 g/L NaCl, 10 g/L tryptone, and 15 g/L Bacto-agar (Difco).

2.3. *Seed Source*

AG DH1012 is the *B. oleracea* genotype described in this chapter. AG DH1012 is a doubled haploid genotype from the *B. oleracea* ssp. *alboglabra* (A12DHd) and *B. oleracea* ssp. *italica* (Green Duke GDDH33) mapping population (13,14). Seed can be obtained from Dr. Graham Teakle of Warwick HRI, University of Warwick, UK

3. Methods

The protocols described below are applicable for *B. oleracea* and *B. napus* transformation and are based on a previously reported method for *B. napus* transformation (15).

3.1. *Seed Sterilization and Germination*

1. Seeds are surface-sterilized in 100% ethanol (BDH) for 2 min, 15% sodium hypochlorite (Fluka, cat. no. 71696) plus 0.1% Tween-20 (Sigma, cat. no. P-9416) for 15 min and rinsed three times for 10 min in sterile distilled water (see Note 4).
2. Seeds are sown on germination medium at a density of 20 seed per 90-mm Petri dish, sealed with Micropore tape, and transferred to a 10°C cold room overnight before being transferred to a 23°C culture room under 16-h day length of 70 $\mu\text{mol}/\text{m}^2/\text{s}$ for 4 d.

3.2. *Agrobacterium Preparation*

1. Prior to inoculation, *A. tumefaciens* strains (see Note 5) are streaked out onto solid LB medium containing the appropriate level of selection. Plates are incubated at 28°C for 48 h.
2. A single colony is transferred to 10 mL of minimal A liquid medium, containing selection and transferred to a 28°C shaker for 48 h.
3. A 50- μL aliquot of the resulting bacterial suspension is transferred to 10 mL of minimal A liquid medium containing no selection and grown overnight in a 28°C

shaker. Overnight suspensions of $OD_{650} = 0.1$ are used for inoculations (dilutions made using minimal A liquid medium).

3.3. Explant Isolation, Inoculation, and Cocultivation

1. Cotyledons are excised from 4-d-old seedlings (**Fig. 2A**; see **Note 6**). This is carried out by gently holding the base of the cotyledon with forceps and slicing through the petiole just above the meristematic region using a sharp scalpel blade (**Fig. 2B**). Care should be taken not to include any meristematic tissue, which does not transform easily and will regenerate “escape” shoots rapidly on selection medium.
2. Once excised, cotyledons are immediately placed onto cocultivation medium in Petri dishes (20×90 mm) ensuring that at least 1 to 2 mm of the cut petiole is implanted into the agar (see **Note 7**; **Fig. 2E**). Ten explants are established on each plate.
3. Once all explants have been isolated, they are inoculated, one at a time, by dipping briefly into an *Agrobacterium* suspension (see **Subheading 3.2.**), ensuring that only the cut end of the petiole is immersed.
4. The cotyledons are then returned to cocultivation plates and sealed with Micropore tape before being transferred to a 23°C culture room under 16-h day length of $40 \mu\text{mol}/\text{m}^2/\text{s}$ for 72 h.

3.4. Selection

1. After cocultivation, cotyledons are transferred to selection medium in deep Petri dishes (20×90 mm; see **Note 8**). Plates are sealed with Micropore tape and returned to the culture room under scattered light. In each experiment it is recommended that two control plates (without kanamycin) also be included, one plate containing explants inoculated with *Agrobacterium*, and the other with explants not inoculated.
2. Explants are transferred to fresh selection medium after 3 wk, with kanamycin levels increased to 25 mg/L at this stage. During this subculture, any white escape shoots are removed. For effective transformation, the cut ends should initiate callus after the first few weeks (see **Note 9**), and shoots develop from this callus after 3 to 5 wk on selection (**Fig. 3**).

3.5. Shoot Isolation

1. When using AG DH1012, the emergence of transgenic (green) shoots can be seen after just 2 wk. After three to five wk, transgenic shoots can be isolated.
2. Green shoots are excised and transferred to 100-mL jars (75×50 mm) containing 25 mL of Gamborg’s B5 medium, 25 mg/L kanamycin, and 500 mg/L carbenicillin. When shoots are initially isolated, it is often not possible to isolate a single meristem. When multiple shoots subsequently develop, further subculturing should be carried out to ensure that a single-stemmed shoot is isolated. This will reduce the number of multistemmed plants transferred to the greenhouse and the likelihood of escapes/chimeras going through. Such plants will complicate the molecular analysis of the primary transgenics.

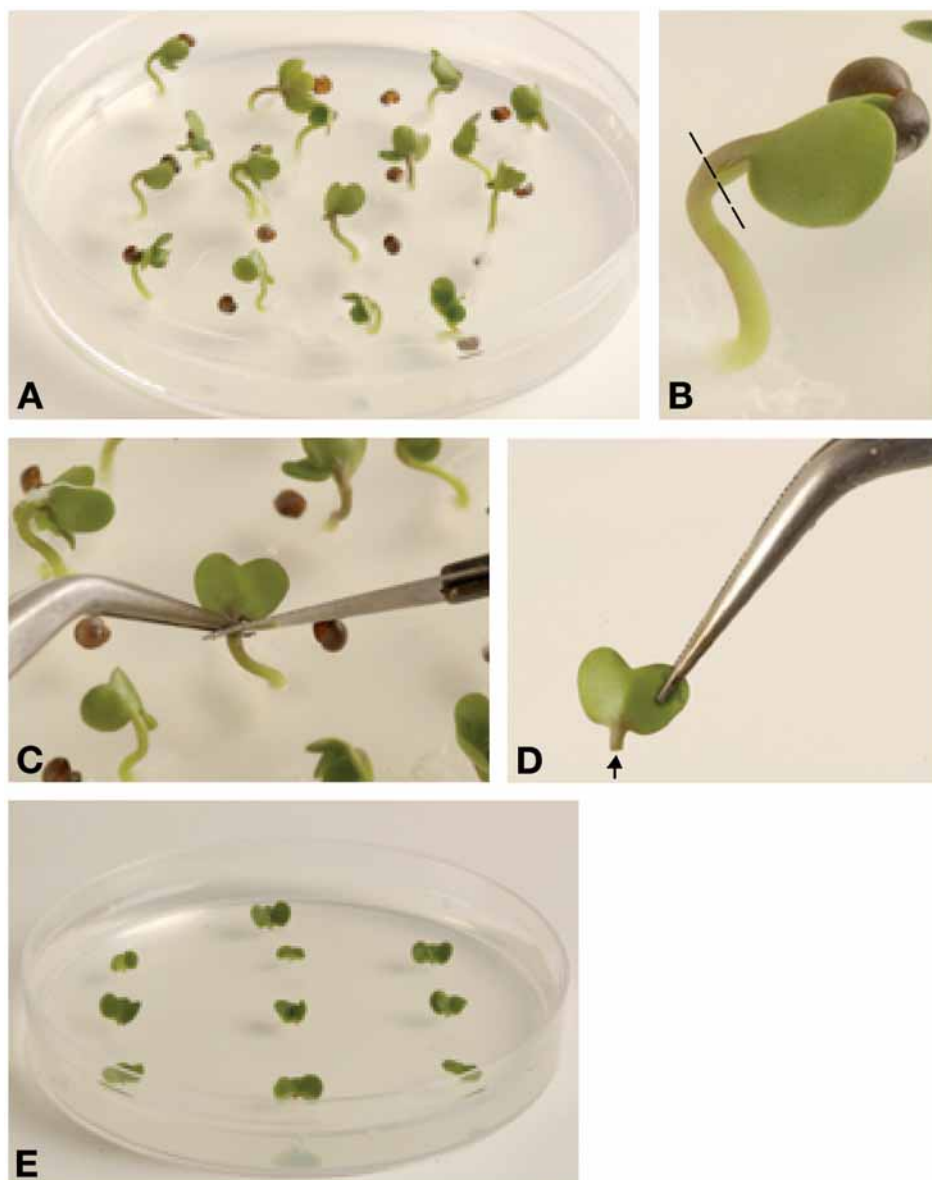


Fig. 2. (A) Four-day-old seedlings. (B) Excision site (dotted line). (C) Explant isolation. (D) Cotyledonary petiole (arrow). (E) Explants on cocultivation medium.

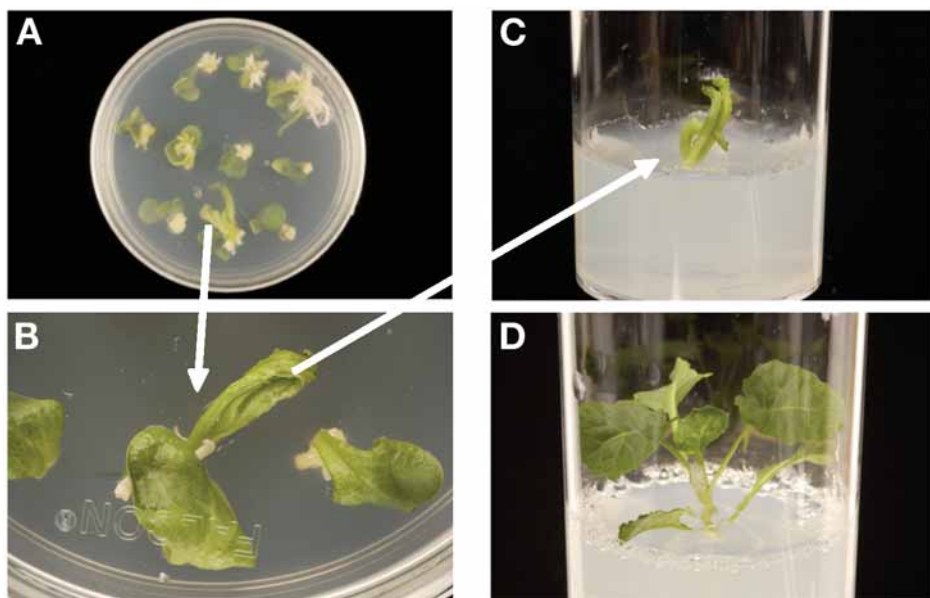


Fig. 3. **(A)** Explants after 3 wk on selection medium. **(B)** Green shoot forming after 3 wk on selection medium. **(C)** Shoot isolation and transfer to Gamborg's B5 medium. Shoots can appear vitrified at this stage. **(D)** Shoot elongation and further development (rooting) stage.

3. Trimmed shoots are maintained on Gamborg's B5 medium (at an increased kanamycin concentration of 50 mg/L), at 23°C under a 16-h day length of 40 $\mu\text{mol}/\text{m}^2/\text{s}$, until roots develop (see **Note 10**).
4. After root elongation (to approximately 20 mm in length), plantlets are transferred to sterile peat pots to allow further root growth before being transferred to the greenhouse.

3.6. Transfer of Plants to Greenhouse

1. Plants are transferred to soil (John Innes No. 2 commercial compost) and maintained under shade within a propagator for the first week. This ensures that plants gradually adjust to reduced humidity and increased light intensity. Greenhouse light conditions are day/night temperatures of $18/12 \pm 2^\circ\text{C}$, 16-h day length, with supplementary lighting ($5.35 \times 10^2 \mu\text{mol}/\text{m}^2/\text{s}$). Plants are fed weekly with a 2:1:1 NPK fertilizer.
2. AG DH1012 is a rapidly cycling genotype and should flower approximately six to eight weeks after transfer. This line is also highly self-compatible and readily sets seed (approx 8–10 wk after bud break) without the need for hand pollination.
3. When in bud, plants are covered with clear, perforated bags (Cryovac [UK] Ltd) to prevent cross-pollination and shaken daily once in flower to encourage seed

set. Pods are allowed to dry on the plant, before being threshed. Seed yield is generally high (>4 g/plant).

4. Notes

1. In *B. oleracea*, tissue culture phenotypes associated with transformability have been identified (7). One of the critical factors for transformation success is the absence of tissue culture blackening (Fig. 1). Genotypes that regenerate shoots from more than 50% of cotyledonary petioles, through a distinct swelling or callus phase and in the absence of blackening, were subsequently found to have higher transformation efficiencies. Transformation efficiency was further improved in genotypes with a high susceptibility to *Agrobacterium*. These phenotypic markers have successfully been applied to *B. napus* material to select genotypes amenable to transformation (8).
2. Medium can be allowed to set and microwaved to remelt if not poured immediately.
3. Alternative selective agents (e.g., hygromycin, phosphinothricin, and others) have not been tested with this protocol; kanamycin is therefore recommended.
4. Typically, 2 g of seed is enough for a 500-explant transformation (depending on seed quality and germination rate). Petri dishes (15 × 50 mm) are ideal for sterilizing seed. Allowing the seed to air-dry will make seed sowing easier. Seed should be placed onto the surface of the medium and not embedded.
5. LBA 4404 is the *Agrobacterium tumefaciens* strain routinely used. AGL 1, EHA 101, and EHA 105 are also suitable strains.
6. When setting up a large experiment, change the scalpel blades frequently. Flame sterilizing will blunt the blade over time. The cut surface of the petiole base is the target tissue for transformation and regeneration. Petiole tissue that is torn or damaged tends not to respond well in culture.
7. This is a good way to determine when cotyledons are the right size/age. If cotyledons have just turned green and can be excised easily (the two cotyledons come away freely without any meristematic tissue), then they are of the right size and age (Fig. 2). The correct size will depend on your genotype, culture room conditions, and other factors. If excised too late, cotyledons will expand rapidly when on regeneration media and not produce shoots.
8. At this stage petioles will have extended and it should be possible to embed them into selection medium and ensure that the cotyledonary lamella is clear of the medium, therefore preventing dieback.
9. Not all genotypes regenerate via a callus phase, but swelling of the cut base should be seen after a week. If extreme blackening to the petiole base occurs, it is likely that the genotype you are working with will be difficult (if not impossible) to transform (Fig. 1A and C).
10. Preliminary molecular analysis (i.e., PCR analysis) can be carried out while shoots are still in vitro. Plant DNA is extracted using the Qiagen DNeasy plant mini kit, from approximately 1 cm² of leaf tissue, and 2 µL of extracted DNA used in a standard 20 µL PCR reaction. For Southern analysis, leaf tissue is collected from plants once they are established in the greenhouse. Plant DNA is

again extracted using Quiagen DNeasy kits with the following modifications: two extractions are carried out for each plant, and the two DNA extractions are combined, ethanol-precipitated, and resuspended in 40 μ L SDW.

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Further illustrative photographs of the transformation of AG DH1012 can be seen on the Biotechnology Resources for Arable Crop Transformation (BRAC) website, www.bract.org. BRAC is funded by Defra.

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Cucumber (*Cucumis sativus* L.)

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Summary

We describe two novel *Agrobacterium tumefaciens*-based methods of cucumber transformation. The first involves direct regeneration from leaf microexplants selected on kanamycin-containing medium. The second involves regeneration from a long-term established embryogenic suspension culture emitting green autofluorescence (GAF) and selection on medium containing hygromycin. In the latter method, GAF was used as a reporter, thereby allowing a simple and reliable identification of transgenic cells with a high regeneration capacity. (No false positives were observed.) The transformation efficiency in the leaf microexplants fluctuated from 0.8 to 6.5% of the primary explants, whereas in the embryogenic suspension-cultured cells it varied from 6.4 to 17.9% of the aggregates. In the GAF method, the step involving the elimination of the *Agrobacterium* cells by antibiotics could be omitted; however, this reduced the transformation efficiency to about 3%. The time required from inoculation to regenerated plant in the greenhouse was the same for both methods, but the GAF method required more preinoculation time than the leaf microexplant method.

Key Words: *Agrobacterium tumefaciens*; leaf microexplant direct regeneration; long-term established embryogenic suspension culture; transformation efficiency; green autofluorescence.

1. Introduction

Agrobacterium-mediated transformation based on plant regeneration from in vitro culture is the most common genetic transformation method used in cucumber. Leaf fragments, petioles, cotyledons, and hypocotyls are the most commonly used explants, although there have been reports of using embryogenic callus or protoplasts (1–6). Research in our laboratory has concentrated on using the original leaf microexplant direct regeneration method (7). The main advantages of this method are fast and efficient regeneration of plants in a wide range of genotypes and the absence of somaclonal variation in traits

determined by nuclear genes. Using this method, we have introduced nine different constructs into the cucumber genome, obtaining almost 100 homozygotic transgenic lines (8,9). False transformants (20%) occurred in only three constructs. Putative transformants ready for growth in a greenhouse were typically obtained within 45 to 60 d after *Agrobacterium* infection, although those transformed with three of the constructs (those containing sequences that encoded tryptophan monooxygenase from *Pseudomonas syringae* pv. *savastanoi*, malate dehydrogenase from *Brassica oleracea*, and apoplastic invertase from *Saccharomyces cerevisiae*) required up to 80 d.

A second method we have developed for cucumber regeneration utilizes a long-term established embryogenic suspension culture characterized by a high regeneration potential and emission of a green autofluorescence (GAF) (10). In the present study, we demonstrate that GAF, which is known to be a marker of regeneration ability (10), can be used as a reporter. We show, that together with resistance to hygromycin, GAF provides a system of double selection of transformants. This double-selection system has several technical advantages, such as an avoidance of false transformants and the expression analysis of a construct both during disorganized proliferation and at early development stages of somatic embryos. Additionally, in the case of less virulent *Agrobacterium* strains, this method does not require the use of antibiotics for bacteria elimination.

In this chapter, we present both methods, as they are complementary. The GAF method (Fig. 1), which allows visual identification of transgenic tissue with high regeneration capacity, is exceptionally useful for promoter function analysis from the early globular to advanced stages of somatic embryogenesis. We demonstrate this method using a construct example of the *gus* (β -glucuronidase) reporter gene fused with different fragments of the *CsXTH1* and *CsXTH3* (xyloglucan endotransglucosylase/hydrolase) (11) or *CsSCR* promoters. At the present time the GAF method is limited by the need to include the *hpt* gene in the construct (kanamycin does not provide a good GAF attenuation effect) and by a reduction in plant fertility (a lower number of seeds is often obtained, and some of the transformed plants are self-sterile, although fertility is mostly restored following crossing with a control). A brief comparison of these two methods is presented in Table 1.

2. Materials

2.1. Seed Materials and *Agrobacterium* Strains

1. Seed source: inbred line cv. Borszczagowski.
2. *Agrobacterium* strains and vectors:

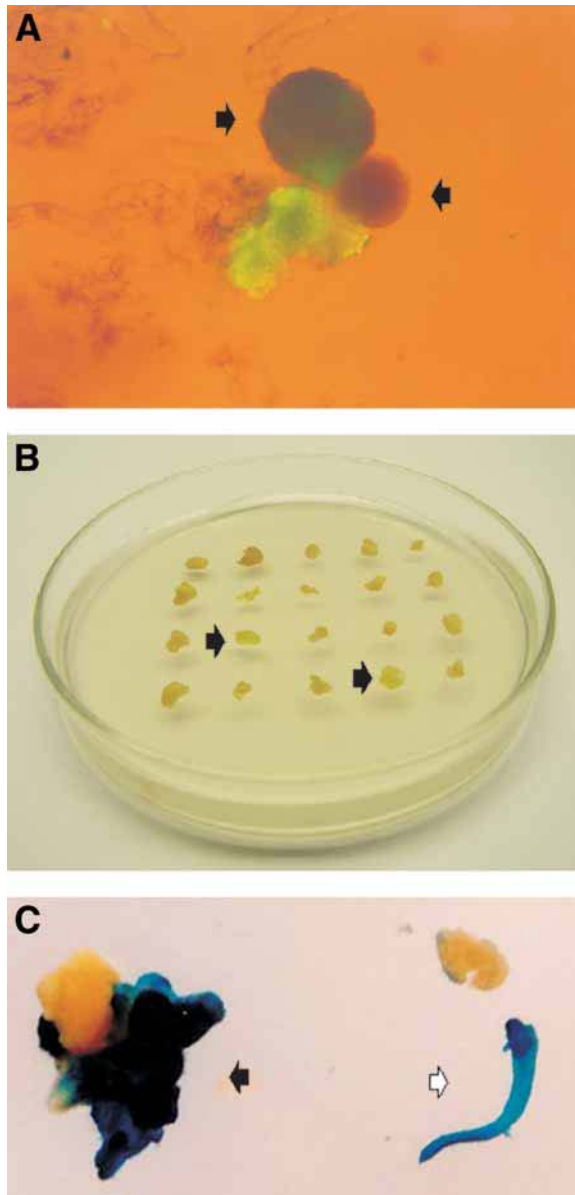


Fig. 1. A GAF-emitting cell suspension transformation method. **(A)** Somatic embryo regeneration from GAF-emitting transgenic suspension; arrows show globular somatic embryos. **(B)** GAF correlates with bright yellow aggregate pigmentation, which makes mass selection of transgenic tissue easy to identify (arrows). **(C)** Histochemical analysis of GUS activity of GAF-germinating aggregate (black arrow) and somatic embryo-emitting aggregate (white arrow).

Table 1
Comparison of Parameters between the Two Transformation Methods:
Leaf Microexplant Direct Regeneration
and GAF in the Borszczagowski Cucumber Line

Parameter	GAF	Leaf microexplant ^a
Time required to obtain transformation-ready explants	13 wk	15–20 d
Selectable agent	Hygromycin	Kanamycin
Screening for transformants	GAF exhibition	Callus growth; shoot regeneration
Selection efficiency (% of transgenics)	100	80–100 ^b
Transformation efficiency (% of primary explants giving transgenic plants)	6.5–17.9 ^c	0.8–6.3 ^b
Plants adapted for greenhouse (% of transferred)	33–70	70–90 ^b
Total number of transgenic lines obtained ^d	12 ^F	100 ^G
Number of successful constructs with transgenic plants	7	9
Self-fertility of T ₀ transgenics (% of plants with seed set)	50	90–100 ^b
Total time (from coculture to plants in the greenhouse)	4–5 mo	3–4 mo ^b

^aAccording to **ref. 7**.
^bDepending on the construct.
^cDepending on *Agrobacterium* strain.
^{dG} As sexual progeny from at least two generations; ^F first sexual progeny.

- a. For the leaf microexplant direct regeneration method, we use strain LBA4404. The vector contains a reporter cassette consisting of the nopaline synthase promoter driving expression of the neomycin phosphotransferase II (*nptII*) coding sequence and various plasmids as background (pUC118, pRUR, pROK2, pBI121, pPCV002) containing coding sequences for thaumatin, tryptophan monooxygenase, dehydrin 10 and 24, malate dehydrogenase, or apoplastic invertase.
- b. For the GAF-emitting cell suspension method, our *Agrobacterium*/vector systems are LBA4404/pCAMBIA1380 and EHA105/pCAMBIA1381z transformation vectors (**12**), the plasmids containing the hygromycin phosphotransferase gene (*hyg*) conferring hygromycin resistance in plant cell, and *gus* as a reporter.

2.2. Plant Media

1. Media for the leaf microexplant method: media for early steps of the transformation procedure are listed in [Table 2](#), and media for later steps are listed in [Table 3](#). These media are Murashige and Skoog (MS)-based media ([13](#)), pH 5.6 to 5.8, established before sterilization. For solidified media, use 7.5 g/L of agar (Difco). Autoclave at 121°C for 17 min.
2. Media for the GAF-emitting cell suspension method: media for this procedure are also MS based and are listed in [Table 4](#); pH 5.6 to 5.8 is established before sterilization. For solidified media use 7.5 g/L of agar (Difco). Autoclave at 121°C for 17 min.

2.3. Other Media, Stock, Solutions, and Supplies

1. Bacterial YEB medium ([14](#)): 5 g/L bovine extract, 1 g/L yeast extract, 5 g/L peptone, 5 g/L sucrose, 0.5 g/L MgSO_4 , pH 7.2 to 7.4 (established before sterilization). For solidified media use 15 g/L of agar (Difco). Autoclave at 121°C for 17 min.
2. Antibiotic stock solutions: 30 mg/mL cefotaxime, 50 mg/mL carbenicillin, and 1 mg/mL hygromycin B. Filter-sterilize and store at -20°C.
3. Acetosyringone (Duchefa): 20 mM stock solution, powder dissolved in 70% ethanol. Filter-sterilize and store at -20°C.
4. Water with Tween-20 (a few drops per liter). Autoclave at 121°C for 40 min.
5. GUS assay solution: the standard buffer containing 1 mg/mL X-gluc (Duchefa) in 100 mM phosphate buffer (pH 7.2) supplemented with the substances enhancing reaction specificity (0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 20 mM EDTA, 0.1% v/v Triton X-100) ([15](#)).
6. Soil mix: sterilize the peat (pH 5.6–5.8) with steam using a closed steel container (6 h) and store for 4 wk under plastic covers in a high standard of phytosanitary conditions. Then add MIS-3 fertilizer (3.5 kg/m³ [Intermas, Poland]) and mix with perlite (3:1, v/v).

3. Methods

3.1. Leaf Microexplant Transformation Method

1. Seed sterilization (the whole procedure stationary): submerge 50 seeds in tap water for 3 h, treat with 50 mL of 10% sodium hypochlorite (15 min), and rinse three times with sterile deionized water (3, 6, and 15 min).
2. Seed germination: place three seeds in one 0.5-L jar containing 80 mL ST0 medium (see [Note 1](#)) closed with a lid (cover) consisting of two sheets of sterilized filter paper and one sheet of aluminum foil. Incubate in a 16-h photoperiod at 23 to 25°C under cool-white fluorescent light of intensity 54 $\mu\text{mol}/\text{m}^2/\text{s}$. Incubate for approx 10 d until they develop one or two true leaves.
3. Explant preparation: harvest from the seedlings only young leaves (0.75–1.0 cm²) that are with folded leaf blade (see [Note 2](#)). Cut the leaves into 2–3 mm² pieces, place them onto ST1 medium for precultivation (2–3 d, 25°C, in the dark, about 50 explants on a single 9-cm Petri dish).

Table 2
Murashige and Skoog Medium (12) Modification Used in the First Steps
of the Transformation Procedure of Borszczagowski Cucumber Inbred Line Leaf Microexplants^a

Components	Medium				
	ST0	ST1	ST1A	ST5	ST6
Macroelements	0.2X NH ₄ NO ₃ ½X 2.5X KNO ₃	1.7X NH ₄ NO ₃ ½X no KNO ₃	1.7X NH ₄ NO ₃ ½X no KNO ₃	1.7X NH ₄ NO ₃ ½X no KNO ₃	1.7X NH ₄ NO ₃ ½X no KNO ₃
Microelements	1X	1X	1X	1X	1X
Vitamins	—	1X	1X	1X	1X
Edamine (mg/L)	—	250	250	250	250
Sucrose (g/L)	10	30	30	30	30
Agar (g/L)	7.5	7.9	7.9	7.9	7.9
2.4-D (mg/L)	—	1	1	1	1
2iP (mg/L)	—	1	1	1	1
Carbenicillin (mg/L)	—	—	—	250	250
Cefotaxime (mg/L)	—	—	—	250	250
Kanamycin (mg/L)	—	—	—	100	150
Acetosyringone	—	—	200 µM	—	—
pH (established before sterilization)	5.8	5.8	5.8	5.8	5.8

^aST2, liquid ST1, pH 5.6; ST3, ST2 with 500 mg/L carbenicillin; ST4, liquid ST5, pH 5.6; ST7, ST6 without kanamycin; ½X, half-strength; 1X, full strength. Add antibiotics and acetosyringone after cooling the medium. Store the filter-sterilized stocks of antibiotics and acetosyringone at –20°C. 0.2X, 0.2 times; 2.5X, 2.5 times.

Table 3
Murashige and Skoog Medium (12) Modification
Used in Later Steps of the Transformation Procedure
of Borszczagowski Cucumber Inbred Line Leaf Microexplants

Components	Medium			
	MSPB	MSRC	1/2EC	2.5EC
Macroelements	1X	½X	½X	½X: 2.5X KNO ₃ 0.2X NH ₄ NO ₃
Microelements	1X	1X	1X	1X
Vitamins	1X	1X	1X	1X
Edamine (mg/L)	250	250	—	—
Sucrose (g/L)	30	20	20	10
Agar (g/L)	7.9	7.9	7.5	7.5
2iP (mg/L)	0.3	—	—	—
IAA (mg/L)	—	—	—	0.5
Carbenicillin (mg/L)	250	250	250	250
pH	5.8	5.8	5.8	5.8

4. Bacteria culture preparation: take a single colony (from freshly prepared culture on solid medium) of the *Agrobacterium tumefaciens* strain and culture in liquid YEB medium containing appropriate concentrations of antibiotics for 2 d at 28°C; shake at 160 to 180 rpm. Then dilute the culture 100-fold in fresh medium and shaker-incubate overnight. Use this culture for explant inoculation if the OD₆₀₀ is between 0.3 and 1.0.
5. Explant inoculation: submerge the explants in a bacteria suspension. Prepare the bacteria suspension in ST2 medium at an OD₆₀₀ of about 0.3 to 0.4 and add acetosyringone during inoculation at a final concentration of 200 µM to the explants-bacteria suspension; gently shake at 90 to 100 rpm, at 28°C, for 20 min.
6. Cocultivation: blot the inoculated explants onto a sterile filter paper and culture on ST1A medium for 2 d, in the dark at 25°C. After cocultivation, wash stationary infected explants in ST3 medium for 90 min and in ST4 medium overnight.
7. Selection: this is a two-step procedure. The first step involves selection on a lower (100 mg/L) kanamycin (from Sigma) concentration (ST5 medium, 25 explants per Petri dish) for 2 wk and the second on a higher (150 mg/L) concentration (ST6 medium). Keep the culture in the dark for 25 to 30 days (25°C) and transfer the explants to a fresh ST6 medium every 2 wk. After 6 to 8 wk of selection, transfer only the fast growing tissues to MSPB shoot induction medium and keep the rest of the callus on ST7 medium for callus propagation.

Table 4
Modifications of Murashige and Skoog Media (12) Used in the GAF Procedure

Procedure step	Medium	Modification of mineral compounds	Modification of organics (mg/L) ^a	Growth regulators (mg/L)	Carbon source (g/L)
Induction of ECS	2.5D + 10E	2.5X KNO ₃ ; no NH ₄ NO ₃	Edamine added (25.0)	2.4-D (1.0)	Sucrose (30)
	V1D1PL	—	Without organics	2.4-D (1.0); 2iP (1.0)	Sucrose (1.5); lactose (30)
Stabilization of ECS; cocultivation and selection	V2BA	—	Without organics	BAP (2.0)	Sucrose (30)
Somatic embryo and plant regeneration	1/2MS	—	—	None	Sucrose (30)
	1/2MS-E(20)	—	Edamine omitted	None	Sucrose (20)

^aVitamins (including m-inositol) and organic nitrogen.

8. Plant regeneration: after 2 wk on MSPB medium, transfer small shoots to MSRC and larger shoots to 2.5 EC medium for shoot elongation. For rooting of 1- to 2-cm-long plantlets/shoots use 2.5EC medium (see **Notes 3** and **4**).

3.2. GAF-Emitting Cell Suspension Transformation Method

1. Initiation of embryogenic cell suspension (ECS): take one apical bud from a 7- to 10-d-old sterile cucumber seedling (obtained as in the microexplant procedure) and place it in 12.5 mL of 2.5D + 10E liquid medium in a 50-mL conical flask on a shaker at 150 rpm (in the dark at 25°C). After 4 wk, cut callusing explant into small fragments and transfer into a fresh medium. Repeat cutting after 3 wk, changing the medium to V1D1PL, and subculture for 3 wk (see **Note 5**). Subsequently, stabilize the ECS in V2BA medium. Keep inoculation density at 0.5 mL packed cell volume (PCV) of tissue per 50 mL of liquid medium, and subculture every 7 d, selecting aggregates with high GAF and compact structure for the passages. For the transformation, take a culture 5 d after the last subculture.
2. *A. tumefaciens* preparation: centrifuge the bacteria from the overnight liquid culture for 15 min at 3920g, remove the bacterial medium, and dissolve the sediment in plant proliferating medium for coculture (V2BA) at OD₆₀₀ = 0.6 (see **Note 6**).
3. Transformation: put 2 mL PCV of tissue into 50 mL of plant medium for coculture (V2BA) in which bacteria were suspended; add 100 µM of acetosyringone and keep the coculture for 3 d on a shaker at 150 rpm, at 25°C, in the dark.
4. Selection: after the coculture, wash the tissue twice using V2BA medium with Tween-20; gently place the aggregates on the surface of solid selective medium V2BA containing 15 mg/L hygromycin (with the addition of cefotaxime and carbenicillin 250 mg/L each or without these antibiotics; see **Note 7**) and place in the dark. Transfer all intact aggregates onto a fresh medium with hygromycin every 2 wk. After three subcultures, select potentially transgenic aggregates based on the presence of GAF, under the microscope, in UV or blue light. Place individual aggregates emitting GAF in a conical flask containing 12.5 mL of liquid proliferation medium V2BA and reinitiate ESC over 2 to 3 wk (see **Note 8**). Maintain ECS for one additional 7-d subculture period (density 0.5 mL PCV per 50 mL of liquid medium).
5. Embryo formation and plant regeneration: during 2 wk perform regeneration of embryos under light in a 100-mL conical flask by transferring approx 0.06 mL PCV transgenic embryogenic tissue into 50 mL ½ MS liquid medium (see **Note 8**); subsequently regenerate plants in jars on ½ MS-E(20) solid medium (transfer no more than 10 germinating embryos per jar).

3.3. Transplanting to Soil and Greenhouse Care

1. Transfer the jars with well-rooted plants (four to five nodes) into the greenhouse and let them stay for approx 4 d (loose the cover to get more external air) then planting into 10 cm (in diameter) pots in sterilized soil mix and cover under the plastic sheet (acclimation for 6–10 d).

2. Replant into 40-cm pots and cultivate in high phytosanitary conditions (16-h photoperiod [prolonged illumination in the spring and autumn is used to extend the photoperiod and to supplement lighting on cloudy days] by sodium lighting [400-W HPS lamps, 200 W/m²], 28°C/20°C [day/night]).
3. Approximately 2 wk after retransplantation, the first pollination can be performed. Self-pollinate by hand to produce the offspring.
4. Seed can be harvested about 8 wk after pollination. In our laboratory, seed set from transgenic plants is similar to the nontransgenic control plants, typically approx 400 per fruit (see **Note 9**).

3.4. Molecular Analyses

1. Molecular analyses such as histochemical GUS assay, PCR, and Southern blotting can be performed at the T₀ plant stage. (We do it after replanting.)
2. For genomic DNA isolation, collect 1 g of fresh leaf tissue and freeze the tissue in liquid nitrogen before storing at -78°C. DNA isolation is performed using the method of Michaels et al. (16) (see **Note 10**). Our typical DNA yield is 10 µg/g of fresh tissue.
3. For progeny analysis of transgenic plant, seeds are planted in jars (12 seeds per jar) containing 80 mL of ST0 medium supplemented with 200 mg/L kanamycin (with the Duchefa product) or 250 mg/L (with the Sigma product). The stock is 50 mg/mL of kanamycin.
4. Seeds are allowed to germinate under a 16-h photoperiod at 23 to 25°C under cool-white fluorescent light of intensity 54 µmol/m²/s, until they develop two true leaves. This takes about 10 d. The leaves of kanamycin-resistant plants should be dark green; kanamycin-sensitive plants should turn pale yellow to white.
5. Transfer the dark green kanamycin-resistant seedlings into 2.5EC medium (without carbenicillin) for 7 to 10 d (for good rooting). Then plants (with four to six nodes) will be moved to the greenhouse (see **Note 11**).

4. Notes

1. The media for seedlings, explant culture, and plant regeneration should be selected according to the cultivars' differences in the requirement of the inorganic nitrogen form (ammonium/nitrate) and auxin susceptibility. (The 2,4-D could be replaced with NAA.)
2. Another critical factor is the leaf stage—it should be a leaf in the phase when it is still folded. Do not take more than three consecutive leaves from one seedling. Depending on the quality of the seed batch, one can remove the seed coat to obtain uniform development of the seedlings if necessary.
3. Ten to 20 independent transformation events may be obtained from 200 leaves; no complete sterility of flowers was ever observed.
4. The production of transgenic plants (able to seed set) is, in our climatic conditions, most efficient when inoculation is performed in the period from January to July.

5. Starting from passage onto V1D1PL, control GAF, selecting tissue with strong fluorescence to the passage. Use an inverted microscope equipped with a fluorescence add-on and UV or blue excitation. The observations do not require tissue removal from the vessels.
6. OD₆₀₀ for coculture depends on bacteria strain. For instance, for EHA105 the best OD₆₀₀ is 0.5, and for LBA4404 the best OD₆₀₀ is 0.7.
7. To eliminate agrobacteria after inoculation, 250 mg/L timentin may be used; it promotes embryo conversion into plant. In the case of a mild strain (LBA4404), washing is sufficient after coculture and the medium with antibiotics is not necessary.
8. For transgenic ESC reinitiation and regeneration, use compact cell aggregates with a high level of GAF (Fig. 1). GAF correlates with bright yellow aggregate pigmentation.
9. The number of seed set in transgenic plants obtained using leaf microexplant method is usually as good as the nontransgenic control, although we observed reduction in seed number (by approx 50%) in some transgenic lines containing constructs such as tryptophan monooxygenase from *P. syringae*, malate dehydrogenase from *B. oleracea*, and apoplastic invertase from *S. cerevisiae*.
10. The method (16) allows one to remove polysaccharides from DNA by ethanol precipitation and therefore is particularly useful for cucumber.
11. Do not keep seedlings in ST0 medium with kanamycin longer than 2 wk. Prolonged culture in this medium will cause poor growing roots and slow development of plants, directly after transferred to the greenhouse.

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Eggplant (*Solanum melongena* L.)

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Summary

Eggplant is an economically important vegetable crop in Asia and Africa, and although it is grown in Europe and the United States, it does not account for a significant percentage of agricultural production. It is susceptible to a number of pathogens and insects, with bacterial and fungal wilts being the most devastating. Attempts to improve resistance through introgression of traits from wild relatives have had limited success owing to sexual incompatibilities. Therefore, a crop improvement approach that combines both conventional breeding and biotechnological techniques would be beneficial. This chapter describes an *Agrobacterium*-mediated transformation protocol for eggplant based on inoculation of seedling explants (cotyledons and hypocotyls) and leaves. We have used this protocol to recover transformants from two different types of eggplant, a *Solanum melongena* L. breeding line, and *S. melongena* L. var. Black Eggplant. The selectable marker gene used was neomycin phosphotransferase II (*nptII*) and the selection agent was kanamycin. In vitro grown transformants acclimated readily to greenhouse conditions.

Key Words: *Solanum melongena*; Black Eggplant; *Solanum*; Solanaceae; vegetable; gene transfer; biotechnology; *Agrobacterium tumefaciens*.

1. Introduction

There are three closely related cultivated species of eggplant: *Solanum melongena* L. (brinjal or aubergine eggplant), *S. aethiopicum* L. (scarlet eggplant), and *S. macrocarpon* L. (gboma eggplant) (1). The brinjal or aubergine eggplant is cultivated throughout the world. Eggplant is a member of the *Solanaceae* family and is closely related to the tomato and potato. It is an autogamous diploid with 12 chromosomes ($2n = 24$).

Eggplant is susceptible to many diseases, nematodes, and insects. It has partial resistance to most of its pathogens; however, these are most often at insufficient levels (2). Attempts at introgression of resistant traits from wild relatives

have had limited success because of sexual incompatibilities (3). Therefore, it will be useful to apply biotechnological techniques such as embryo rescue, somatic hybridization, and *Agrobacterium*-mediated transformation in combination with conventional breeding to develop crop improvement programs. In addition to these approaches, molecular genetic linkage maps and other genomics resources are being generated for eggplant, which will provide the information necessary for identification, localization, marker-assisted selection, and isolation of qualitative and quantitative traits in eggplant (4).

Eggplant has been shown to be amenable to tissue culture and there are several reports on plant regeneration via organogenesis (5) and somatic embryogenesis (6). The earliest report of *Agrobacterium*-mediated transformation of eggplant was by Guri and Sink in 1988 (7). Since that time there have been several other reports of *Agrobacterium*-mediated transformation primarily for the purpose of generating transgenics that contain the *Bacillus thuringiensis* endotoxin (BT) gene for insect resistance (8). Explants used for regeneration and transformation studies have included leaves and seedling explants (5–8) in addition to roots (9).

The protocol outlined in this chapter is based on information we learned from reviewing the literature in combination with our experience in transformation of solanaceous crops. For this *Agrobacterium*-mediated method, we used seedling explants (cotyledons and hypocotyls) and leaves from an *S. melongena* breeding line and *S. melongena* var. Black Eggplant. The seeds were germinated under sterile in vitro conditions as a source of material for transformation. We used the neomycin phosphotransferase (*nptII*) gene as a selectable marker and kanamycin as a selection agent. The transformation efficiency (percent of explants that gave rise to transgenics that rooted on kanamycin-containing rooting medium) for these two eggplant types was 10%; however, transformation efficiency can vary for different eggplant lines. The main problem we encountered was vitrification (10), and strategies we applied to remedy this problem are discussed.

2. Materials

2.1. *Agrobacterium tumefaciens* Strains and Selectable Markers

We have used *A. tumefaciens* LBA4404 and the neomycin phosphotransferase II (*nptII*) gene as a selectable marker (see Note 1).

2.2. Culture Media for *Agrobacterium tumefaciens*

1. 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). Autoclave, and then cool to 55°C before adding the appropriate selection agent (depending on the vector).

2. YM medium: 400 mg/L yeast extract, 10 g/L mannitol (Fisher Scientific), 100 mg/L NaCl, 200 mg/L $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$ (Sigma, St. Louis, MO). Autoclave, and then cool to 55°C before adding the appropriate selection agent (depending on the vector).

2.3. Tissue Culture

1. Eggplant seeds (*see* **Note 2**).
2. Surface sterilants: 70% (v/v) ethanol and 20% (v/v) commercial bleach solution with 0.1% (v/v) Tween-20 (Fisher Scientific).
3. $\frac{1}{2}$ MSO medium: 2.15 g/L MS salts (cat. no. MSP0501, Caisson Laboratories, Rexburg, ID), 2 mg/L thiamine-HCl (Sigma), 0.5 mg/L pyridoxine-HCl (Sigma), 0.5 mg/L nicotinic acid (Sigma), 10 g/L sucrose (PhytoTechnology Laboratories, Shawnee Mission, KS), 8 g/L Agar-Agar (Sigma), pH 5.8.
4. 2,4-Dichlorophenoxyacetic acid (2,4-D) (Sigma): 1 mg/mL stock. Dissolve the powdered chemical in a few drops of 1 M KOH and then add purified water (Milli-Q purification system, Millipore, Billerica, MA) to volume. Store at 4°C up to 3 mo.
5. Kinetin (Sigma): 1 mg/mL stock. Dissolve the powder in a few drops of 1 M HCl and then add purified water to volume. Store at 0°C indefinitely.
6. Zeatin (PhytoTechnology Laboratories): 1 mg/mL stock. Dissolve the powder in a few drops of 1 M HCl and then add purified water to volume. Filter-sterilize the stock and store in 1-mL aliquots in sterile 1.5-mL-capacity Eppendorf tubes at 0°C.
7. Glycine (Sigma): 2 mg/mL stock. For a 50 mL stock: 100 mg glycine. Filter-sterilize the stock and store at 4°C up to 1 mo.
8. B5 vitamins. for 50 mL 1000X stock: 5 g myoinositol (Sigma), 0.5 g thiamine-HCl, 50 mg nicotinic acid, 50 mg pyridoxine-HCl.
9. KCMS medium: 4.3 g/L MS salts, 100 mg/L myoinositol, 1.3 mg/L thiamine-HCl, 0.2 mg/L 2,4-D, 200 mg KH_2PO_4 (Fisher Scientific), 0.1 mg/L kinetin, 30 g/L sucrose, 5.2 g/L Agargel (Sigma), pH 5.5. Dispense 25 mL into 100 × 20-mm Petri dishes. Store in the dark at room temperature for 1 mo.
10. KCMS liquid medium: as per KCMS medium (**item 9**), but do not add the Agargel.
11. Tobacco NT1 suspension culture: to initiate culture, transfer approximately 1.8 g of friable callus to 50 mL of KCMS liquid medium in a sterile 250-mL Erlenmeyer flask. Grow on a gyratory shaker at 100 rpm in the dark (24°C) and subculture every 7 d by transferring 2 mL of the old suspension to 48 mL of KCMS liquid medium.
12. MSO-2% liquid medium: 4.3 g/L MS salts, 100 mg/L myoinositol, 0.4 mg/L thiamine-HCl, 20 g/L sucrose, pH 5.8. Store at room temperature for up to 1 mo.
13. Kanamycin monosulfate: 50 mg/mL stock. Place 2.5 g kanamycin monosulfate (PhytoTechnology Laboratories) in a beaker with a magnetic stir bar and 35 mL deionized water. Allow kanamycin to dissolve. Bring final volume to 50 mL with deionized water. Filter-sterilize and divide into 1-mL aliquots. Store at 0°C for up to 6 mo.

14. Timentin (PhytoTechnology Laboratories): 100 mg/mL stock. Dissolve the powder in purified water and then filter-sterilize. Store as 3-mL aliquots at 0°C.
15. MS(E)/75kan/300timentin medium: 4.3 g/L MS salts, 100 mg/L myoinositol, 0.4 mg/L thiamine-HCl, 20 g/L sucrose, 8 g/L Phytablend (Caisson Laboratories), pH 5.6. Autoclave, and then cool to 55°C before adding 1 mg/L zeatin, 2 mg/L glycine, 300 mg/L timentin, and 75 mg/L kanamycin. Dispense 25 mL into 100 × 20-mm Petri dishes. We pour approximately 40 plates per liter.
16. Sterile, coupled Magenta boxes: two Magenta boxes attached together by a Magenta coupler (PhytoTechnology Laboratories, cat. no. C937). Autoclave the boxes after they are connected with the couplers.
17. ½ G(E)/50kan/200timentin medium: 1.54 g Gamborg's salts (PhytoTechnology Laboratories), 1 mL of B5 vitamins 1000X stock, 9 g/L Phytablend, pH 5.6. Autoclave, and then cool to 55°C before adding 200 mg/L timentin and 50 mg/L kanamycin. Dispense 50 mL into each coupled Magenta box system.
18. Micropore tape: size ½-inch wide (McKesson, cat. no. 1530-0).
19. Vented Magenta box lids (PhytoTechnology Laboratories, cat. no. C160).
20. Sterile paper towels.
21. Sterile distilled water.
22. Sterile metal spatulas with spoon on one end (VWR International, S. Plainfield, NJ, cat. no. 57952-253).
23. Transfer to soil: 10.16- or 15.24-cm pots (4- to 6-inch pots), soil-less potting mix (not commercially available; 538.6 g Unimix + III, Griffin Greenhouse and Nursery Supplies, Tewksbury, MA; 2.27 kg lime, Hummert International, Earth City, MO; 0.161 m³ peat moss, Hummert International; 0.34 m³ vermiculite, Hummert International; 2.27 kg Osmocote 17-7-12, Grower Supply Inc., Forest Hill, LA) (see **Note 3**), cover plants with clear plastic bags or containers such as Magenta boxes or plastic cups.

3. Methods

3.1. Seed Germination

1. Immerse seeds in 70% (v/v) ethanol for 30 s with gentle agitation.
2. Using a pipet, remove the 70% ethanol and discard.
3. Add a volume of the 20% commercial bleach and Tween-20 solution large enough to cover the seeds completely. Agitate gently for 20 min. Using a pipet, remove the bleach solution and discard.
4. Rinse seeds with sterile distilled water by agitating for 1 min and then remove the rinse water. Repeat two more times.
5. Transfer seeds onto several layers of sterile paper towels to remove the excess rinse water.
6. Transfer seeds onto ½ MSO medium for germination in Magenta boxes (6 cm long × 6 cm wide × 10 cm high) (see **Note 4**). Fifteen to 20 seeds can be cultured per box. Incubate at 25°C under lights at a 16-h light/8-h dark photoperiod. Germination time varies with the eggplant line being used (see **Note 5**).

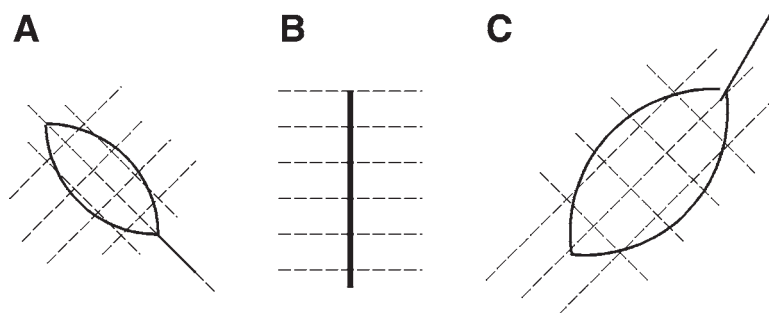


Fig. 1. Preparation of eggplant explants for transformation. Cuts were made along the dashed lines. Only explants with cut edges all around were inoculated with *Agrobacterium*. Discard outer edges of cotyledons and leaves. **(A)** Cotyledons. **(B)** Hypocotyls. **(C)** Leaves.

3.2. *Agrobacterium* Preparation

1. Day 1: streak *Agrobacterium* from a glycerol stock onto LB + antibiotic selection appropriate for vector used. Incubate at 28 to 30°C for 2 d (see **Note 6**).
2. Day 3: transfer three to four well-formed *Agrobacterium* colonies from the plate into a 50-mL culture of YM medium + antibiotic selection appropriate for the vector used.
3. Place 50 mL cultures onto a shaker/incubator for 24 h (or until culture has reached an OD₆₀₀ of 0.5).

3.3. Explant Preparation

1. Day 2: prepare tobacco NT-1 feeder layer on KCMS medium in Petri plates (100 × 20 mm). Place 2 mL of a 7- to 10-d-old liquid NT-1 cell suspension culture onto the center of the media surface of a KCMS (solid) Petri plate. Cover cells with a 7-cm sterile filter paper. Replace cover of plate. Return plates to original plastic sleeve and seal sleeve with tape. Culture NT-1 feeder layer plates in an upright position in the dark for 24 h at 24°C.
2. Day 3: cut eggplant cotyledons, hypocotyls, and leaves into explants (**Fig. 1**) (see **Note 7**). Only use explants with cut edges all around and discard outer, uncut edges. Size of explants should be between 0.5 and 1 cm.
3. Place 50 explants per Petri plate. Place cotyledon and leaf explants adaxial (top) side down, in contact with the NT-1 feeder layer. Seal plates with Micropore tape (½-inch wide).
4. Place into a growth room with the following specifications: photoperiod, 16 h/8 h, 70 μmol/m²/s, 24°C.

3.4. *Agrobacterium* Inoculum

1. Day 4: after the OD₆₀₀ of the *Agrobacterium* culture has been determined to be 0.5, spin in a centrifuge at 8221g for 10 min.

2. Pour off supernatant and discard.
3. Resuspend pellet in MS0-2% liquid medium at the same original volume as the overnight culture by vortexing. The *Agrobacterium* is now ready to use for transformation.

3.5. Inoculation of Explants and Cocultivation

1. Day 4: using a sterile metal spatula, gently scrape explants from the filter paper into a sterile Magenta box. Transfer explants from one Petri plate (50 explants) per box. Pipet 20 to 25 mL of prepared *Agrobacterium* inoculum over the explants. Gently agitate the box periodically to stir explants with the *Agrobacterium*.
2. After 5 min, pipet *Agrobacterium* out of the Magenta box, and transfer back to prepared inoculum to be used for remaining explants (see **Note 8**). Using a sterile metal spatula, remove inoculated explants to a sterile paper towel to blot off excess *Agrobacterium*.
3. Return all explants to NT-1 feeder layer for a 2-d coculture. Place leaf and cotyledon explants adaxial (top) side down onto the feeder layer. Culture 50 explants per Petri plate. Seal plates with Micropore tape ($\frac{1}{2}$ -inch wide). Incubate at 25°C under lights at a 16-h light/8-h dark photoperiod.

3.6. Maintenance of Cultures and Regenerated Shoots

1. Day 6: transfer explants to MS(E)/75kan/300timentin medium. Culture 25 explants per Petri plate. Place explants abaxial (bottom) side down in contact with medium. Seal plates with Micropore tape (size $\frac{1}{2}$ inch). Return culture to the same incubation conditions at 25°C under lights at a 16-h light/8-h dark photoperiod. Repeat transfer to MS(E)/75kan/300timentin medium every 3 wk until all shoots are harvested.
2. When a small callus develops on explants, transfer explants to Magenta boxes with vented lids (see **Note 9**). Culture six explants per box.
3. When callus is large enough to be removed from the explants, transfer one or two calli to MS(E)/75kan/300timentin medium per Magenta box with vented lid (see **Note 10**).
4. Harvest shoots as they begin to emerge, and transfer shoots to $\frac{1}{2}$ G(E)/50kan/200 timentin medium for rooting in coupled Magenta box system (see **Note 11**). Harvest only one shoot/callus. Discard callus once a shoot has been harvested from it (see **Note 12**).
5. Shoots can take between 4 and 5 wk to begin rooting. Once a shoot has rooted on the first round of rooting, it should go to a second round of rooting on $\frac{1}{2}$ G(E)/50kan/200timentin medium. Once the shoot has rooted a second time on this medium, it is ready to go to the greenhouse (see **Note 13**). The overall percentage of plants with roots after the first round of rooting is 75%.
6. Continue transferring callus to fresh MS(E)/75kan/300timentin medium every 3 wk, harvesting shoots as they emerge.

3.7. Transfer to Soil

1. Gently remove plants with well-formed root systems from the culture vessel and wash the medium from the roots using tepid water (see **Note 14**).
2. Transfer each plantlet to a 10.16- or 15.24-cm pot (see **Note 5**) containing thoroughly wetted soil-less potting mix, and cover with a plastic container or plastic bag secured to the pot (see **Note 15**).
3. Transfer plants to a growth chamber; shaded area of a greenhouse, or laboratory setting with lights (see **Note 16**). The light intensity should be approximately 45 $\mu\text{mol}/\text{pm}^2/\text{s}$ with a photoperiod of 16-h light and 8-h dark.
4. After 1 wk, cut a small hole in the plastic bag or raise the plastic container a small amount. Repeat this each day for 1 wk for a gradual acclimation. Remove the cover on the 8th d. The percent of plants that survive acclimation is 100%.
5. If plants were maintained in a laboratory setting, they will need a gradual acclimation to greenhouse conditions. They should be grown in a shaded area of a greenhouse for several days before transferring to an area of full light. Plants were fertilized twice per week with Peter's Excel 15-5-15 Cal-Mag (Griffin Greenhouse and Nursery Supplies, cat. no. 67-2160).

4. Notes

1. The strain LBA4404 is our preferred strain, although it is recA positive and constructs must be checked regularly for recombination events. The only selectable marker gene used was *nptII* along with kanamycin at 75 mg/L as the selection agent for transformants and 50 mg/L for selective rooting. The selectable marker gene and the appropriate level of selection agent for your system can be substituted.
2. We have used this protocol to recover transgenic lines from an *S. melongena* L. breeding line and *S. melongena* L. var. Black Eggplant.
3. Recipe for soil-less potting mix in U.S. measurements: 1 lb, 3 oz Unimix + III (Griffin Greenhouse and Nursery Supplies), 5 lbs lime (Hummert International), 5.7 cu. ft. peat moss (Hummert International), 12 cu. ft. vermiculite (Hummert International), 5 lbs Osmocote 17-7-12 (Grower Supply).
4. A small percentage of the eggplant seeds had a low level of an internal bacterial contaminant. Therefore, we incorporated 300 mg/L timentin into subsequent batches of germination medium after autoclaving. This treatment was effective in preventing the contaminant from growing.
5. Germination time for our seeds ranged from 2 to 4 wk.
6. It is recommended to include an empty vector control whenever a transformation experiment with a new gene of interest is initiated. The empty vector control will allow you to determine if a gene of interest in a construct has a negative effect on transformation efficiency (percent of explants that result in transformants). In addition, plant material from an empty vector control could also be used as negative controls when material from a gene of interest construct is analyzed.

7. Our seedlings explants were 3 to 5 wk old when explants were taken. At this stage, most of the seedlings had at least two leaves.
8. A longer period of incubation may cause water-soaked damage to cells.
9. Vitrification of shoots can be a problem with eggplant grown under in vitro conditions. We found that using the vented lids allowed better air exchange and prevented vitrification.
10. We observed less vitrification of regenerants when a small number of calli (one to two per Magenta box) were cultured. For large calli, we cultured only one per box.
11. Coupled Magenta boxes provided better aeration for the plants and decreased the incidence of vitrification.
12. We harvested only one shoot/callus to avoid harvesting sister clones (transformants that arise from the same transformation event). This is to ensure that each transgenic plant is an independent transformation event.
13. Internal *Agrobacterium* contamination can sometimes occur in transformants. It is strongly recommended that before transformants are transferred to the greenhouse, they be cultured on rooting medium without timentin for at least 1 mo. Alternatively, PCR can be performed with primers specific for *Agrobacterium*. Plants with *Agrobacterium* contamination should be discarded because the contaminant cannot be eradicated.
14. Washing the medium from the roots reduces the chance of adverse bacterial and fungal growth that may kill the plantlet once it is placed in soil.
15. We use plastic cups, old Magenta boxes, or clear plastic juice containers that have the tops cut off. It is important to cover each plant immediately after transfer to soil to prevent wilting.
16. If new transfers to soil are placed in direct sunlight, heat will build up under the cover and kill the plant.

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Lettuce (*Lactuca sativa* L.)

Ian S. Curtis

Summary

Lettuce is a globally important leafy vegetable with the United States being the largest world producers. The crop is susceptible to a number of viruses that are aphid transmitted and also highly vulnerable to post harvest diseases. Although wild species of lettuce are an important source of disease resistance genes, their introgression into commercial lettuce has been limited owing to sexual incompatibilities. Hence, the development of a gene transfer system for lettuce would be extremely valuable both in improving the genetic diversity of the crop and also for the transfer of useful agronomic traits. This chapter describes an *Agrobacterium*-mediated gene delivery system that is highly adaptable for the production of transgenic plants using a wide range of lettuce germplasms. The system described, commonly referred to as the *genotype-independent transformation system*, has been used for the transfer of several agriculturally useful traits into commercial varieties of lettuce. In this case, *A. tumefaciens* strain LBA4404 carrying a binary vector with supervirulent pToK47 was used for infecting excised cotyledonary explants. The plant selectable marker gene neomycin phosphotransferase II (*nptII*) was used, and transformed plants were selected using kanamycin in the culture medium. The β -glucuronidase gene with intron (*gus*-intron) was also used in the gene transfer study to confirm the transgenicity of regenerated plants further.

Key Words: *Agrobacterium*-mediated gene transfer; β -glucuronidase; *Lactuca sativa* L.; lettuce; neomycin phosphotransferase II; transgenic plants.

1. Introduction

Lettuce (*Lactuca sativa* L.) is a high-value leafy vegetable that is grown globally. It is an efficient inbreeding species that belongs to the family Asteraceae (formerly known as a member of the *Compositae*). Cultivated lettuce belongs to the *Serriola* group (**1**) along with *L. saligna*, *L. virosa*, *L. serriola*, and *L. altaica* FISCH. and MEY, which are diploid with nine chromosomes ($2n = 18$).

The main breeding aims of lettuce are disease and insect resistance, high yield, uniformity, high quality, and improved shelf-life. Although the genetic diversity of lettuce can be improved through sexual crosses with closely related wild species (*L. sativa* × *L. serriola* only), cross-pollination is inherently difficult owing to the structure of the flower. The generation of genetic variability in lettuce has been achieved through mutation breeding (2), somaclonal variation (3), and protoplast fusion/somatic hybridization (4–6), but such procedures are largely inefficient and time consuming.

Since many of the breeding objectives for lettuce improvement involve single gene traits, the use of *Agrobacterium*-mediated gene transfer is highly desirable. Lettuce explants are highly responsive in tissue culture to a wide range of culture media (7), and regenerated shoots have been recorded for many genotypes (8). The first report on the production of transgenic lettuce plants was achieved by infecting excised seedling cotyledons with *Agrobacterium tumefaciens* (9). Thereafter, other research groups targeted the cotyledon as the ideal explant for transformation studies owing to its ability to produce large numbers of transformed shoots and the ease with which such explants can be generated (10,11), but such systems were only efficient for few genotypes. Since breeding objectives vary for different lettuce genotypes, a gene transfer system that could be applied to a large number of lettuce germplasms would be highly beneficial for the future improvement of the crop.

The transformation protocol described is more commonly referred to as the genotype-independent transformation system for lettuce (12). This system has been successfully used in the production of transgenic plants from more than 40 cultivars/breeding lines of lettuce (including wild species). Such a protocol involves the infection of cotyledon explants with a supervirulent *A. tumefaciens* strain 1065 (12) and utilizing a highly efficient tissue culture system, as described by Webb (13). In this study, the selectable marker neomycin phosphotransferase II (*nptII*) gene was used to allow transformed plants to be screened on medium containing kanamycin monosulfate. In addition, a second marker (reporter gene), the β -glucuronidase (*gus*)-intron gene was also used in transformation studies to confirm the transgenicity of regenerated shoots/rooted plantlets further by GUS histochemical staining. Factors that can influence the number of transformed plants such as the presence of a nurse culture, the concentration of bacterial inoculum, and the concentration of selection agent (kanamycin) were all evaluated in optimizing the yield of transformed plants. The transformation efficiency (defined as the percentage of explants that gave rise to transgenics that rooted on medium containing kanamycin monosulfate and gave a positive GUS stain throughout the manuscript) ranged between 16 and 97% (variety ‘Lake Nyah’ being one of the most responsive toward

Agrobacterium-mediated gene transfer). The problems encountered in transgene stability over several generations are discussed.

2. Materials

2.1. *Agrobacterium tumefaciens* Strain, Plasmids, and Marker Genes

A. tumefaciens strain LBA4404 (14) harboring the pBIN19 derivative pMOG18 (15) with supervirulent pToK47 (16; see Note 1). The binary vector, also known as pVDH65, carried the *nos-nptII-nos* and CaMV 35S-us-intron-CaMV 35S gene constructs between the left and right borders of the T-DNA, respectively. The resulting *A. tumefaciens* strain is commonly known as 1065 (see Note 2).

2.2. Culture Medium for *A. tumefaciens*

1. Luria broth (LB) medium: 10 g/L Bacto-tryptone (Becton Dickinson, Sparks, MD), 5 g/L yeast extract (Becton Dickinson), 10 g/L NaCl, 14 g/L Bacto agar (Becton Dickinson), pH 7.0. Autoclave at 121°C for 20 min, and then cool to 50 to 55°C in a waterbath before adding the antibiotics.
2. Rifampicin (Sigma): 4 mg/mL stock. Dissolve powder in methanol and then filter-sterilize through a 0.2 µm membrane (Minisart, Epsom, UK). Store at –20°C for 2 to 3 mo.
3. Kanamycin monosulfate (Sigma): 10 mg/mL stock. Dissolve powder in purified water (Mill-Q purification system, Millipore, Billerica, MA), sterilize by filtration, and store at –20°C for 2 to 3 mo.
4. Tetracycline-HCl (Sigma): 5 mg/mL stock. Dissolve powder in Mill-Q water and then sterilize by filtration. Store at 4°C for 1 to 2 mo (see Note 3).
5. Liquid LB medium: LB medium without agar but containing 50 mg/L kanamycin sulfate, 40 mg/L rifampicin, and 2 mg/L tetracycline-HCl.
6. Semi-solid LB medium: LB medium with 1.4 g/L agar, 50 mg/L kanamycin sulfate, 100 mg/L rifampicin, and 5 mg/L tetracycline-HCl.

2.3. Tissue Culture

1. Lettuce seeds cv. ‘Lake Nyah’ were supplied by Dr. D. A. C. Pink (Horticulture Research International, Wellesbourne, UK). Cultivars that also give high transformation efficiencies (70–85%) include ‘Lobjoits’ and ‘Evola’ and can be acquired from the seed company Leen de Mos, ‘s-Gravenzande, P. O. Box 54-2690 AB, The Netherlands.
2. Surface sterilants: 10% (v/v) “Domestos” bleach (Lever Fabergé, Kingston-upon-Thames, UK).
3. Germination medium: 2.15 g/L Murashige and Skoog (MS; 17) salts and vitamins (Flow Laboratories), 10 g/L sucrose, 8 g/L agar (Sigma), pH 5.9. Dispense 20 mL into 9-cm-diameter Petri dishes. Plates are sealed with Micropore tape and then stored at room temperature for up to 4 wk in the dark.

4. UM medium for callus induction (**18**): 4.71 g/L MS salts and vitamins, 30 g/L sucrose, 2 g/L casein hydrolysate, 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D; Sigma), 0.25 mg/L kinetin, 9.9 mg/L thiamine-HCl, 9.5 mg/L pyridoxine-HCl, 4.5 mg/L nicotinic acid, 8 g/L agar (Sigma), pH 5.8. For liquid medium the agar is not added. Dispense and store the medium as described for germination medium.
5. α -Naphthalene acetic acid (NAA, Sigma): 1 mg/mL stock. Dissolve powder in a few drops of absolute ethanol and add Mill-Q water to volume. Store at 4°C for 1 to 2 mo.
6. 6-Benzylaminopurine (BAP; Sigma): 0.5 mg/mL stock. Dissolve 50 mg of powder in 1 mL of 1 N HCl and then gradually make up to volume with Mill-Q water. Store at 4°C for 1 to 2 mo.
7. Cefotaxime (Claforan, Roussel Laboratories, Wembley, UK): 10 mg/mL stock. Dissolve powder in Mill-Q water and then sterilize by filtration and store at -20°C for 2 to 3 mo.
8. Carbenicillin (Beechams Research Laboratories, Brentford, UK): 100 mg/mL stock. Dissolve powder in Mill-Q water and then sterilize by filtration and store at -20°C for 2 to 3 mo.
9. Shoot regeneration medium: 4.71 g/L MS salts and vitamins, 30 g/L sucrose, 0.04 mg/L NAA, 0.5 mg/L BAP, 500 mg/L carbenicillin, 100 mg/L cefotaxime, 50 or 100 mg/L kanamycin monosulfate, 8 g/L agar (Sigma), pH 5.8. Store the medium in a coldroom (4–6°C) for up to 4 wk (20 mL/plate; 40 mL/175 mL-capacity screw-capped Powder Round glass jar (Beatson Clarke, Rotherham, UK)).
10. Root inducing medium: 4.71 g/L MS salts and vitamins, 30 g/L sucrose, 50 or 100 mg/L kanamycin monosulfate (see **Note 4**), 2.5 g/L Phytigel (Sigma), pH 5.8 (see **Note 5**). Dispense medium into Powder Round jars (40 mL) or Magenta GA7 boxes (Sigma; 90 mL) and store as for shoot regeneration medium.
11. In the glasshouse, plants are grown in 9-cm-diameter plastic pots containing a mixture (3:3:2; v/v/v) of Levington M3 compost (Fisons, Ipswich, UK), John Innes No. 3 compost (J. Bentley, Barrow-on-Humber, UK), and perlite (Silvaperl, Gainsborough, UK).
12. Selection medium: 2.15 g/L MS salts and vitamins, 10 g/L sucrose, 200 mg/L kanamycin monosulfate, 8 g/L agar (Sigma), pH 5.9. Dispense medium into 9-cm-diameter Petri dishes (20 mL) and store in a coldroom for up to 4 wk.

All media are sterilized by autoclaving (121°C, 20 min). Stock solutions of antibiotics are added to the medium after autoclaving. (For agar-containing medium, antibiotics are added at 50–55°C.)

2.4. GUS Histochemical Solution

1. GUS substrate mixture: 10 mM Na₂EDTA, 0.1 M phosphate buffer (pH 7.0), 0.1% Triton X-100, 0.5 M K₃Fe(CN)₆, 1 to 5 mM X-gluc. The substrate X-gluc is dissolved in ethylene glycol monoethyl ether (EGMGE) as a 1 mg/mL stock prior to use; store for a maximum period of 14 d at -20°C.

3. Methods

3.1. Seed Germination

1. Soak seeds in 10% (v/v) “Domestos” for 30 min with gentle agitation.
2. Remove sterilant with a pipet and discard. At this stage, all culture work is undertaken in a laminar air hood under sterile conditions.
3. Rinse seeds twice with sterile distilled water.
4. Transfer seeds onto germination medium (40 seeds/dish) and grow under a light intensity of $18 \mu\text{mol}/\text{m}^2/\text{s}$ (16-h photoperiod, daylight fluorescent tubes) at $23 \pm 2^\circ\text{C}$ for 7 d (see **Note 6**).

3.2. *Agrobacterium* Preparation

1. Day 1: streak *Agrobacterium* from a glycerol stock onto LB agar medium containing antibiotics. Incubate cultures in the dark at 28°C for 2 d.
2. Day 3: transfer one loopful of bacteria to 10 mL of LB liquid medium containing antibiotics and incubate overnight at 28°C on a shaker (dark conditions) until culture has reached an $\text{OD}_{600\text{nm}}$ of 1.0 (see **Note 7**).

3.3. Explant Preparation

1. Day 4: soak a single sterile 7-cm-diameter Whatman filter paper in liquid UM medium and place onto the surface of the UM agar medium (with or without nurse culture; see **Note 6** for nurse culture preparation).
2. Carefully excise the cotyledons (petioles intact) from 7-d-old seedlings but avoiding the apices located near the base of the petiole (see **Note 8**). Make shallow cuts (see **Note 9**) on the abaxial surface of the cotyledon with a sharp scapel blade and then place onto filter paper (wounded-side down) overlying the UM agar (with or without nurse culture) prior to infection with *Agrobacterium* (8–10 explants/plate; see **Note 10**).

3.4. *Agrobacterium* Inoculum

1. Day 4: once the $\text{OD}_{600\text{nm}}$ of the *Agrobacterium* culture has reached 1.0, the culture is ready for inoculating the explants.
2. Remove 2 mL of culture, transfer to 20 mL of UM liquid medium contained in a 9-cm Petri dish, and gently swirl to mix (see **Note 11**).

3.5. Inoculation of Explants and Cocultivation

1. Day 4: remove the excised cotyledons from the UM agar plates and float, with their scored surface in contact with the *Agrobacterium* inoculum for 10 min (see **Note 12**). Control explants (uninoculated) are scored and floated on liquid UM medium.
2. Using forceps, carefully transfer the inoculated explants to a sterile filter and blot dry. When excess inoculum has been removed, transfer the explants back to the UM agar plates (see **Note 13**). Place a single sterile filter paper over the explants to keep the leaves flat. Seal plates with Micropore tape. Cocultivate the explants for 2 d under the same conditions as for germinating seeds.

3.6. Maintenance of Cultures and Shoot Regeneration

1. Day 6: transfer explants to shoot regeneration medium (8–10 explants/dish). Place explants abaxial side down and gently submerge the petiole ends of the cotyledons to a depth of 2 mm into the medium. Seal plates with Micropore tape. Incubate cultures as for germinating seeds and subculture to fresh medium every 14 d.
2. Approximately 7 d after inoculating the explants with *Agrobacterium*, it is a good idea to select one to two explants per dish for GUS activity to check that transformation was successful. Immerse explants in assay buffer and incubate in the dark at 37°C (minimum period 6 h, but usually overnight). Remove tissues from the buffer, immerse in 95% ethanol (1–2 h), and then transfer the explants to 70% ethanol for 2 to 3 d. Several transfers of explants into fresh 70% ethanol may be necessary to remove all chlorophylls efficiently. GUS activity in explants can be checked using a stereomicroscope. Explants not used for GUS staining should show shoot development approx 35 d after inoculation.
3. Excise shoots (approx 1 cm in length) from the explant using a sharp scalpel blade and transfer to root inducing medium (one to three shoots/jar or Magenta box; see **Note 14**). Culture the shoots at $20 \pm 2^\circ\text{C}$, 12-h photoperiod and light intensity of $18 \mu\text{mol}/\text{m}^2/\text{s}$ (daylight fluorescence tubes). Roots should become visible 7 to 10 d after inserting shoots into the medium. To confirm GUS activity in the kanamycin-resistant plantlet, both leaf pieces and root segments (approx 0.5 cm in length) should be stained for GUS as described earlier (see **Subheading 3.6.2.**).

3.7. Transferring Plants to Soil and Plant Acclimation

1. Gently remove rooted, GUS-positive plants from the culture vessel and carefully wash away the medium from the roots using tepid tap water (see **Note 15**).
2. Transfer each plantlet to a 9-cm-diameter plastic pot containing a soil mix. Stand the potted plants in trays and water. Enclose each plant with a clear polythene bag and incubate in a glasshouse away from direct sunlight ($23 \pm 2^\circ\text{C}$, 16-h photoperiod, and light intensity of $29 \mu\text{mol}/\text{m}^2/\text{s}$; daylight fluorescence tubes).
3. After 7 d, remove one corner from each bag and then remove the other corner 7 d later (see **Note 16**).
4. Remove the entire bag from each potted plant after 21 d. Transfer the plants to the glasshouse ($24 \pm 2^\circ\text{C}$, 16-h photoperiod, and light intensity of $80 \mu\text{mol}/\text{m}^2/\text{s}$) and grow to maturity (see **Note 17**).
5. Plants are given a general liquid fertilizer (containing NPK) at 14-d intervals.
6. The time to bolting and flowering from transplanting plants into pots depends on the type of lettuce grown. Butterhead varieties of lettuce such as 'Luxor' and 'Flora' bolt and reach anthesis before crispheaded types such as 'Lake Nyah' and 'Saladin' (butterheads, approx 80 d to bolting, 95 d to anthesis; crispheads, approx 115 d to bolting, 130 d anthesis).
7. Seeds can be harvested approx 5 to 6 wk after anthesis (seed yield is approx 100–250 seeds per plant).

4. Notes

1. The presence of pToK47 in *A. tumefaciens* strain LBA4404 in the transformation of cotyledonous explants of lettuce improves the yield of transformed shoots per explant. However, Southern blot analysis of transformed plants produced from treatments involving the supervirulent plasmid generally carry more than one copy of the transgene compared with treatments without the plasmid.
2. Recent studies on the stability of gene expression in several generations of transgenic lettuce (19,20) revealed that a truncated pea plastocyanin promoter 784 bp in length (–784 bp petE; 21) was superior compared with CaMV 35S. The CaMV 35S promoter appears to be vulnerable to DNA methylation, which subsequently results in transgene silencing. Therefore, it is advisable to substitute the CaMV 35S promoter sequence for the petE promoter if stable gene expression is required over several generations.
3. After 4 wk, the stock solution can precipitate but should maintain its original color. Once the solution starts to become darker yellow, the solution should be discarded and a fresh stock prepared.
4. Kanamycin monosulfate used at a concentration of 100 mg/L is more efficient in selecting transformed shoots compared with 50 mg/L.
5. Using Phytigel at 2.5 g/L as a gelling agent in the root inducing medium allows plantlets to develop better root systems compared with culture media containing 8 g/L agar.
6. The transformation efficiency of some cultivars of lettuce can be improved if nurse cultures are employed (9). If nurse cultures are to be used, 3 mL of a log-phase cell suspension of *Nicotiana plumbaginifolia* (9) or albino *Petunia hybrida* cv. ‘Comanche’ (22) is to be pipeted onto agar-solidified medium and cultured as for seeds, 2 d before being used as a nurse culture. In the case of *P. hybrida* cell suspensions, cultures are maintained in 80 mL liquid UM medium contained in 250-mL Erlenmeyer flasks on a horizontal shaker (80 rpm, 16-h photoperiod, 18 $\mu\text{mol}/\text{m}^2/\text{s}$, $22 \pm 2^\circ\text{C}$). During subculture, decant the majority of spent medium to waste and then pour approx 15% of the dense cell suspension into 250-mL flasks containing 80 mL of fresh medium. Then 3-mL aliquots of log-phase cell suspension (taken 5 d after subculture) is transferred onto UM agar-solidified medium and used as a nurse culture plate.
7. When setting up an overnight liquid culture of *A. tumefaciens* strain LBA4404, it is important to try to suspend the loopful of bacteria into the medium. To achieve this, the bacteria should be brushed against the inside of a wetted surface of the vessel using the loop. Depositing large lumps of bacteria into the liquid medium often produces poorly suspended overnight cultures, which are less efficient in transformation studies.
8. To minimize the chance of experimental error, it is advisable to select cotyledons of approximately equal size for between and within experiments.
9. Cuts should be performed transversely across the surface of the cotyledon. Cutting through the explant can lead to stress to the leaf and possible cell death.

Shallow wounds act as sites for *Agrobacterium* infection and can greatly improve the chance of an explant being successfully transformed and ultimately improve the yield of transformed plants.

10. The number of explants per dish depends on the size of the cotyledons used in the study. As a guide, the cultivars 'Lake Nyah' and 'Lobjoits', which produce long, narrow cotyledons, up to 10 explants per dish can be used. However, for cultivars such as 'Bastion', which produce relatively wide cotyledons, a maximum of eight cotyledons should be used per dish.
11. It is not necessary to spin down the overnight culture of *Agrobacterium* prior to preparing the inoculation medium. The concentration of rifampicin in the 1:10 (v:v) dilution in the infection medium is too low to have a significant effect on the transformation of explants.
12. Increasing the inoculation time beyond 10 min can lead to explant stress. For this reason, in terms of improving the yield of transformed plants, a long incubation time should be avoided.
13. Overdrying of explants should be avoided, as this can cause explant death. Once residual inoculum has been removed from an explant, it should be transferred immediately to the culture medium.
14. Shoots should be excised from explants when a stem is visible (approx 3 mm in length). Excising a shoot in a rosette stage of development (no stem) can result in poor root development when culture in a root induction medium.
15. To improve the chance of a plant from tissue culture acclimatizing to conditions inside a glasshouse, the plant should have a well-developed root system and the leaves of the plant should not exhibit signs of nutrient deficiency such as chlorosis. In addition, the culture medium should be washed away efficiently from roots to reduce the chance of bacterial and fungal infection, which can severely limit the survival of a newly transferred plant to soil.
16. To reduce the chance of fungus attack on the plant, it is advisable to remove surface condensation inside the bag by gently tapping the pot at daily intervals.
17. In some cases, plants grown in pots form a closed network of leaves or "heart." To allow efficient seed production from such plants, these leaves should be gradually eased apart to expose the apical shoot without causing leaf damage. Breakage of such leaves often results in fungal attack at wounded sites, which can ultimately cause plant death.

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Tomato (*Lycopersicum esculentum*)

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Summary

Tomato (*Lycopersicum esculentum*) is an important fruit crop in the Americas, southern Europe, the Middle East, and India, with increasing production in China, Japan, and Southeast Asia. It is amenable to producing pharmaceuticals, particularly for oral delivery; for many of the same reasons, it is a popular vegetable. Its fruit does not contain toxic substances and is palatable uncooked; it is easily processed; the plants are able to be propagated by seed or clonally by tip or shoot cuttings; the plants have a high yield of fruit; there is reasonable biomass and protein content; and they are easily grown under containment. This chapter describes *Agrobacterium*-mediated transformation of the tomato nucleus using cotyledons as explants. We have used this protocol to generate transgenic lines from several tomato cultivars expressing various genes of interest and selectable markers. We also provide protocols for molecular characterization of transgenic lines and batch processing tomato fruit.

Key Words: Tomato nuclear transformation; *Agrobacterium*-mediated transformation; fruit processing; molecular characterization of transformed lines; plant-made vaccines.

1. Introduction

Tomato has been categorized in many ways including as a weed; a beautiful, poisonous plant; a tax-avoiding fruit; and a taxable vegetable. Despite its initial uncertain classification, tomato has become the most widely grown vegetable in the United States and the second most commonly grown vegetable in the world. There are over 4000 tomato varieties differing in disease resistance, fruit characteristics, growth habitats, maturity date, and plant size, yet programs still strive to produce a better product. Some programs use stable transformation of the tomato nucleus to introduce discrete fragments of DNA containing genes that confer desirable traits. A wide variety of traits such as

resistance to environmental stress (1–3), disease resistance (4–6), and genes improving agronomic traits (7,8) have been stably transformed into the tomato genome. The biotechnology industry has also used tomato to produce plant-made pharmaceuticals (9–14).

The tomato plant is a good species for producing orally delivered pharmaceuticals for many of the same reasons it is a popular vegetable. Its fruit does not contain toxic substances and it is palatable uncooked; it can be propagated by seed or clonally by shoot cuttings; the plants have a high fruit yield; there is reasonable biomass and protein content; and tomato plants are easily contained and grown in greenhouses. Plant-made pharmaceuticals have faced several challenges during their development, particularly when the transgenic plant material is used for oral delivery. The difficulties have historically included consistency and concentration of doses and stability of the pharmaceutical in plant tissues. The last difficulty is particularly relevant to tomato because of the perishable fruit. We have circumvented these problems when investigating tomato as a production and delivery system for pharmaceuticals by minimal processing. Freeze-dried fruit can be formulated as a pharmaceutical batch with consistent, high concentration of the pharmaceutical of interest, stable at room temperature for at least 1 yr. This chapter describes the protocols we use for stable, *Agrobacterium*-mediated transformation of tomato using cotyledons as explants; characterization of resultant transgenic lines; and batch processing of fruit for a stable, concentrated, consistent batch of the protein of interest. Transformation efficiency varies with different cultivars, selectable markers, and introduced genes; however, using our protocol, 10 to 14% of cotyledon explants give rise to plants that test positive by Southern analysis.

2. Materials

2.1. *Agrobacterium tumefaciens* Strains and Selectable Markers

Our transformation protocol supports a number of variables including the tomato line (see **Note 1**), *Agrobacterium tumefaciens* strain (see **Note 2**), and the selectable marker (see **Note 3**). The binary plasmids containing the T-DNA and plant expression cassettes are electroporated into *Agrobacterium* as described in Chapter 3.

2.2. Culture Media for *Agrobacterium tumefaciens*

1. Luria-Bertani (LB) medium: 10 g/L Bacto-tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L Bacto agar. Autoclave, and then cool to 55°C before adding the appropriate selective agent (depending on the vector).
2. YM medium: 400 mg/L yeast extract, 10 g/L mannitol (Fisher Scientific), 100 mg/L NaCl, 200 mg/L MgSO₄·7H₂O (Sigma, St. Louis, MO). Autoclave,

and then cool to 55°C before adding the appropriate selective agent (depending on the vector).

3. Selective agent: prepare the appropriate agent according to concentration and solubility, and then filter-sterilize (see **Note 3**).

2.3. Plant Materials

1. Seeds of *Lycopersicon esculentum* lines (see **Note 1**).
2. Tobacco NT1 suspension culture: to initiate culture, transfer about 1.8 g of friable callus to 50 mL of KCMS liquid medium (see **Subheading 2.4.**) in a 250-mL Erlenmeyer flask. Grow on a gyratory shaker at 100 rpm in the dark and subculture every 7 d by transferring 2 mL of the old suspension to 48 mL of KCMS liquid medium.

2.4. Tissue Culture and Greenhouse

1. Surface sterilant: 20% (v/v) commercial bleach solution with 0.1% (v/v) Tween-20.
2. $\frac{1}{2}$ MSO medium: 2.15 g/L MS salts (cat. no. M524, PhytoTechnology Laboratories, Shawnee Mission, KS), 2 mg/L thiamine-HCl, 0.5 mg/L pyridoxine-HCl, 0.5 mg/L nicotinic acid, 10 g/L sucrose, 8 g/L Agar-Agar, pH 5.8.
3. KCMS medium: 4.3 g/L MS salts, 100 mg/L myoinositol, 1.3 mg/L thiamine-HCl, 0.2 mg/L 2,4-D, 200 mg KH_2PO_4 , 0.1 mg/L kinetin, 30 g/L sucrose, 5.2 g/L Agargel, pH 5.5. Dispense into 100 $\times$ 20-mm Petri dishes. Store in the dark at room temperature for 1 mo.
4. KCMS liquid medium: as per KCMS medium, but do not add the Agargel.
5. MS liquid medium: 4.3 g/L MS salts, 2 mg/L glycine, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine-HCl, 0.4 mg/L thiamine-HCl, 0.25 mg/L folic acid, 0.05 mg/L d-biotin, 30 g/L sucrose, pH 5.6. Store at room temperature for up to 1 mo.
6. 2Z selective medium: 4.3 g/L MS salts, 100 mg/L myoinositol, 1 mL/L Nitsch vitamin stock (1000x), 5.2 g/L Agargel, pH 6.0. Autoclave, and then cool to 55°C before adding 2 mg/L zeatin, 300 mg/L timentin, and the appropriate selective agent. Pour into 100 $\times$ 20-mm Petri dishes. We pour approximately 40 plates per liter.
7. 1Z selective medium: prepare as per 2Z selective medium; however, use only 1 mg/L zeatin. Allot about 25 mL into 100 $\times$ 20-mm Petri dishes or 50 mL into Magenta boxes [6 cm long $\times$ 6 cm wide $\times$ 10 cm high].
8. Selective rooting medium: 4.3 g/L MS salts, 1 mL/L Nitsch vitamins stock, 30 g/L sucrose, 8 g/L Bacto agar, pH 6.0. Autoclave, and then cool to 55°C before adding 300 mg/L timentin and the appropriate selection agent.
9. 2,4-Dichlorophenoxyacetic acid (2,4-D): 1 mg/mL stock. Dissolve the powdered chemical in a few drops of 1 M KOH and then add purified water (Milli-Q purification system) to volume. Store at 4°C up to 3 mo.
10. Kinetin: 1 mg/mL stock. Dissolve the powder in a few drops of 1 M HCl and then add purified water to volume. Store at 0°C indefinitely.

11. Zeatin: 1 mg/mL stock. Dissolve the powder in a few drops of 1 M HCl and then add purified water to volume. Filter-sterilize the stock and store in 1-mL aliquots in sterile 1.5-mL-capacity Eppendorf tubes at 0°C.
12. Nitsch vitamins: for 50 mL 1000x stock: 100 mg glycine, 500 mg nicotinic acid, 25 mg pyridoxine-HCl, 25 mg thiamine-HCl, 25 mg folic acid, 2 mg D-biotin. Dissolve by adjusting to pH 7.0 and store at 0°C up to 1 yr.
13. Selection agent: as appropriate for the selectable marker found in the gene construct (see **Note 3**).
14. Timentin: 100 mg/mL stock. Dissolve the powder in purified water and then filter-sterilize. Store as 3-mL aliquots at 0°C (see **Note 4**).
15. Sterile paper towels.
16. Magenta box: size of 6 cm long $\times$ 6 cm wide $\times$ 10 cm high.
17. Plant pots: size of 10.16 cm (or 4 inch) or 15.24 cm (or 6 inch) in diameter.
18. Soil mix: soil-less potting mix (not commercially available). Mix 538.6 g (or 1 lb 3 oz) Unimix + III (Griffin Greenhouse and Nursery Supplies, Tewksbury, MA), 2.27 kg (or 5 lb) lime (Hummert International, Earth City, MO), 0.161 m³ (or 5.7 ft³) peat moss (Hummert International), 0.34 m³ (or 12 ft³) vermiculite (Hummert International), and 2.27 kg (or 5 lbs) Osmocote 17-7-12 (Grower Supply Inc., Forest Hill, LA).

2.5. Molecular Characterization

1. Crude protein extraction buffer: 50 mM sodium phosphate, pH 6.6, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 10 μ g/mL leupeptin, 1 mM phenylmethane-sulfonyl fluoride (PMSF).
2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) gel loading buffer: 300 mM Tris-HCl, pH 6.8, 600 mM dithiothreitol (DTT), 12% SDS, 0.6% bromophenol blue, 60% glycerol.
3. Tris-glycine buffer: 25 mM Tris, 250 mM glycine, pH 8.3, 0.1% SDS.
4. PBSTM: 1% dry milk in phosphate-buffered saline (PBS) + 0.1% Tween-20.
5. PBST: PBS + 0.1% Tween-20.
6. DNeasy Plant kit (Qiagen, Valencia, CA).

3. Methods

3.1. Preparation of *Agrobacterium* Culture

1. Four days before transformation, streak the *Agrobacterium* strain containing the binary plasmid of interest onto plates of LB medium containing the appropriate selection agent. Incubate at 28°C for 48 h.
2. Two days before transformation, select a well-formed *Agrobacterium* colony to initiate a 50-mL culture in YM selective medium. Maintain at 28°C in a shaking incubator at 250 rpm until the culture reaches an OD₆₀₀ of 0.4 to 0.6. Should the culture overgrow this OD₆₀₀, dilute the culture until the reading is below 0.5, and then grow for about 1 h before rechecking the OD₆₀₀.
3. On the day of transformation, centrifuge the culture at 5510g for 10 min at 20°C before resuspending the pellet in 50 mL MS liquid medium.

3.2. Preparation of Plant Materials for Transformation

1. Six to 8 d before transformation, sterilize tomato seeds by immersing them in 20% (v/v) commercial bleach solution for 10 min, and then rinse three times with sterile distilled water (see **Note 5**).
2. Using sterile forceps, place 25 seeds in each Magenta box containing 50 mL of 1/2 MSO medium. Germinate at $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$, under lighted conditions using cool and warm fluorescent bulbs (F40CW and F40WW; Philips Lighting Co., <http://www.lighting.philips.com/index.htm>) and a 16-h light/8-h dark regime at $45 \mu\text{mol}/\text{m}^2/\text{s}$.

3.3. Preparation of Tobacco NT1 Feeder Layer and Explants

1. Two days before transformation, use a 10-mL wide-bore pipet to dispense 2 mL of a 7-d-old (7 d after subculture to fresh KCMS liquid medium) NT1 suspension culture onto KCMS medium. Swirl the plate gently to distribute the cells evenly around the center of each plate (see **Note 6**).
2. Gently place a sterile Whatman filter paper (7-cm circle) over the plated NT1 cells (**Fig. 1A**), seal the plates using Micropore tape, and incubate overnight in darkness at $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$.
3. The day before transformation, cut the cotyledons from the seedlings (**Fig. 1B**). *If the first true leaves have emerged from the seedlings, discard them.* Should the cotyledons be longer than 1 cm, cut the cotyledons in half crosswise (see **Notes 7 and 8**).
4. Place the top side (adaxial) down on the filter paper covering the NT1 cells. Up to 80 explants can be cultured per feeder layer (**Fig. 1C**).
5. Seal the plates with Micropore tape and incubate overnight at $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$, under 16-h light/8-h dark at $45 \mu\text{mol}/\text{m}^2/\text{s}$.

3.4. Transformation, Selection, and Regeneration

1. On the day of transformation, transfer the cotyledon explants from the feeder layer plates to 25 mL of *Agrobacterium* in liquid MS medium (see **Subheading 3.2.**) in a sterile wide-mouthed container. Incubate for 5 min (see **Note 9**).
2. Remove *Agrobacterium* by pipeting the culture from the container.
3. Transfer the explants onto sterile paper towels, gently blot to remove excess *Agrobacterium*, and place top side down onto the original feeder layer plates (**Fig. 1D**).
4. Culture in darkness at 19 to 25°C for 48 h cocultivation (see **Note 10**).
5. Two days after cocultivation, place 25 cotyledon explants top side (adaxial) up on 2Z selective plates.
6. Seal plates with Micropore tape and culture at $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$ using 16-h light/8-h dark at $45 \mu\text{mol}/\text{m}^2/\text{s}$.
7. Transfer infected explants to 1Z selective medium every 3 wk, culturing only 10 explants per plate (see **Note 11**).
8. Regeneration of shoots from callus should occur about 8 to 10 wk after transformation (**Fig. 1E**).

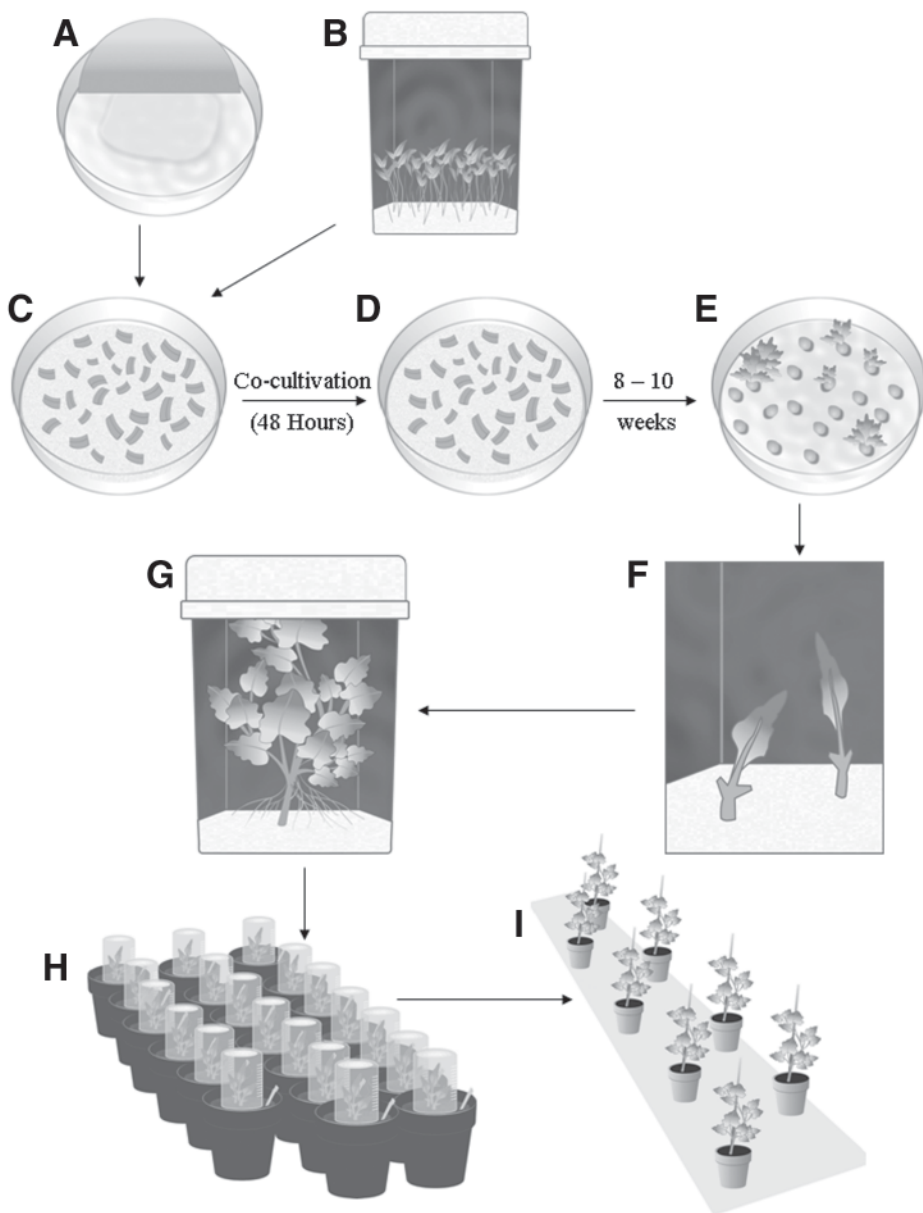


Fig. 1. *Agrobacterium*-mediated transformation of tomato. **(A)** Prepare NT1 cell feeder plates (9 cm diameter) 24 h in advance of **(B)** excising the cotyledons of 6- to 8-d-old seedlings. **(C)** Plate the 1-cm cotyledons upside down onto the plates and let recover for 24 h. **(D)** Cocultivate the explants with the *Agrobacterium* for 48 h and then transfer to selection medium. **(E)** Shoots regenerate 8 to 10 wk after transformation. **(F)** Excise shoots from callus when they are about 2 cm tall and transfer to rooting medium. **(G)** Transfer plantlets to soil once a well-established root system has developed and they are about 9 cm tall. **(H)** Transfer to 15.24-cm pots and **(I)** acclimatize to greenhouse conditions.

9. When shoots are about 2 cm tall, excise from callus and transfer to 50 mL of selective rooting medium in Magenta boxes (**Fig. 1F**). Grow at $24^{\circ}\text{C} \pm 1^{\circ}\text{C}$ under 16-h light/8-h dark at $45 \mu\text{mol}/\text{m}^2/\text{s}$ (see **Note 12**).
10. Shoot tip and nodal cuttings should be made every 3 to 4 wk. Plantlets should be transferred to fresh selective rooting medium depending on the rate of growth, but at least two additional transfers should be spent on selective rooting medium. Possible internal contamination of plantlets with *Agrobacterium* can be detected by not adding timentin to the rooting medium just before transfer to the field or greenhouse (see **Note 13**). The selective agent may also be excluded at this time if desired.

3.5. Transfer to Soil and Harvest

1. Gently remove plants with well-formed root systems from the culture vessel, and wash the medium from the roots using tepid water (see **Note 14**).
2. Transfer each plantlet to a 10.16- or 15.24-cm pot (see **Note 15**) containing a thoroughly wetted soil-less potting mix and cover with a plastic container or plastic bag secured to the pot (**Fig. 1H**).
3. Transfer plants to a growth chamber; shaded area of a greenhouse, or laboratory setting with lights (see **Note 16**).
4. After 1 wk, cut a small hole in the plastic bag or raise the plastic container a small amount. Repeat this each day for 1 wk for gradual acclimation (**Fig. 1I**). Remove the cover on the 8th d.
5. If plants were maintained in a laboratory setting, they will need a gradual acclimation to greenhouse conditions. They should be grown in a shaded area of a greenhouse for several days before transferring to an area of full light.
6. We submit new plantlets to a misting regimen (see **Note 17**); however, we have successfully acclimatized plantlets without this step using the above procedure.
7. Once plants reach approximately 30 cm in height, pot in 21.6-cm-diameter pots using soil-less mix. Gently fix the plants to stakes and train a straight upright stem. Ensure that plants are accurately labeled. We water daily or place drip emitters in each pot. Drip emitters control plant nutrition. Approximately 1 L of “fertigation” is supplied each day. The standard fertilizer mix for tomato is 200 ppm Peter’s Excel 15-5-15 CalMag. We mainly use physical barriers and biocontrol to manage pests, but pesticides are also used (see **Note 18**).
8. Once the plants outgrow the stakes (1 m), give the tomato support by attaching the growing tip to a string held overhead on a horizontal cable. Add clips weekly to guide the tomato up the string support.
9. Harvest the T_0 fruit (see **Note 19**). As the fruit is perishable, collect the seeds as soon as possible. However, a prompt harvest of seeds may not be possible with large fruit harvests. If this is the case, store the fruit at approximately 24°C with a low relative humidity (30–40%) for up to 2 wk.
10. Collect the T_1 seeds by cutting the fruit in half through the blossom end and scraping the seeds and jelly-like parenchyma onto a paper towel (see **Note 20**). In most cases, seed set in transgenic fruit does not differ from wild type. Spread

the seeds over the surface of the towel to reduce the amount of parenchyma on the seeds.

11. Transfer the seeds onto a 15.24 × 15.24-cm weigh paper (VWR Scientific, West Chester, PA) and allow to dry for 2 d at room temperature.
12. Store the seeds still attached to the weigh paper at 4°C in appropriately labeled envelopes.

3.6. Analysis of the T_1 Generation

3.6.1. Selective Agent Screening

1. Germinate the T_1 seeds as per **Subheading 3.2.**, but add the appropriate selection agent to the MS media (see **Note 21**).
2. Record the number of seeds that germinated to determine whether Mendelian inheritance is being followed (see **Note 22**).
3. Transfer the seedlings to soil as per **Subheading 3.4**.
4. Molecular analysis of the plantlets can occur when the growth of the plantlets will not be endangered as a result of collecting tissue.

3.6.2. DNA Extraction for PCR and Southern Analysis

1. Using the DNeasy Plant kit, extract the genomic DNA from transgenic and nontransgenic (control) leaf tissue. Reduce the volume of buffers for smaller amounts of material.
2. Perform PCR reaction with Taq DNA polymerase and the appropriate primers. Always include water and wild-type genomic DNA as negative controls. Run PCR products on an agarose gel and examine for a band of the expected size.
3. Southern analysis is usually performed on PCR-positive lines to establish whether the transgene has been integrated into the plant's genome and if so, verify the number of insertion sites and transgene copy number. Analyze 15 to 20 µg of DNA as described by Sambrook et al. (15) (see **Note 23**).

3.6.3. Protein Extraction for Western and ELISA Analysis

3.6.3.1. CRUDE PROTEIN EXTRACTS

1. Add an appropriate volume of ice-cold extraction buffer to plant material (see **Note 24**).
2. Homogenize tissue in a QBiogene (Carlsbad, CA) Fast Prep machine.
3. Centrifuge at 36,375g in an Eppendorf 5415D microcentrifuge at 4°C for 5 min to pellet insoluble materials.
4. Collect the supernatant, keep on ice during analysis, and then store at -80°C.

3.6.3.2. WESTERN ANALYSIS

1. Western analysis can determine whether the protein of interest is the correct size, forms oligomers or polymers, and is glycosylated.
2. Add 30 µL of each crude protein sample (see **Note 25**) to 6 µL of 6X SDS gel loading buffer (see **Note 26**), boil for 10 min, and then place on ice.

3. Centrifuge the samples at 26,725g for 5 min at 4°C in an Eppendorf 5415D microcentrifuge. Load into a 12% SDS polyacrylamide gel, and run in Tris-glycine buffer at 30 mA per gel until the dye front reaches approximately 5 mm from the end of the gel.
4. Using a Bio-Rad Trans Blot Cell (50 V for 2 h), transfer the separated proteins in the gel to a polyvinylidene difluoride (PVDF) membrane.
5. Block the membrane using PBSTM for 1 h at room temperature with gentle agitation or slow revolution in a Hybaid oven (Ashford, Middlesex, UK).
6. Rinse the membrane twice at room temperature in PBST before incubating with the primary antibody diluted to the appropriate concentration in PBSTM.
7. Rinse the membrane in PBST and then incubate for 1 h with slow rotation in an appropriate dilution of secondary antibody conjugated with a marker such as horseradish peroxidase (HRP).
8. Rinse the membrane in PBST and wash once for 15 min and three times for 5 min. Detect signal using the Amersham ECL + kit as per the manufacturer's instructions (see **Note 27**).

3.6.3.3. ELISA ANALYSIS

1. The type of enzyme-linked immunosorbent assay (ELISA) used to determine the concentration of a specific protein in a crude protein extract depends on the reagents available to you, specifically on the type of antibodies available. Crowther (16) and Bruyns et al. (17) are good sources for determining which protocol to use and how to optimize it.

3.7. Batch Processing Tomato Fruit

We classify plant lines as “elite” when they contain at least 10 µg/g fresh weight of the protein of interest in the correct conformation and have a low transgene copy number (<3).

1. Clonally propagate elite lines under tissue culture conditions using stem cuttings on rooting medium or in the greenhouse using shoot cuttings dipped in any rooting hormone product (see **Note 28**) prior to transfer to soil. Allow to mature and harvest the T₀ fruit.
2. Autoclave and dispose of any overripe fruit, fruit with signs of insect, bacterial, or fungal infection, or fruit with extensive blossom end rot (a physiological abnormality resulting in superficial disfigurement of fruit).
3. Immediately wash fruit in a 1% bleach solution, rinse with dionized or reverse-osmosis water, place into sealed autoclave bags within a sealable box, and transport to the processing site (see **Note 29**).
4. Remove seeds as described in **Subheading 3.3**.
5. Cut the fleshy fruit tissue into 1- to 1.5-cm slices, place in plastic bags, seal, weigh, and store at -20°C (see **Note 30**).
6. Freeze-dry the fruit for a minimum of 85 h at a maximum shelf temperature of 20°C. The materials can be removed from the freeze-drier for very short periods during this time to break up any large pieces and ensure full drying (see **Note 31**).

7. Vacuum-seal in Federal Drug Administration (FDA)-approved 4-mm polyethylene bags (separate for multiple batches), crush the contents by hand or mechanical means, and store in a dry environment at room temperature until the entire harvest has been freeze-dried (see **Note 32**).
8. Soak a food-grade metal sifter in 10% bleach for 10 min, rinse with deionized water, and allow to air-dry.
9. To reduce the freeze-dried fruit to fine powder, sift, using the prepared food-grade sifter in a fume hood. Visually inspect the powder for homogeneity.
10. Autoclave and dispose of any material that does not pass through the sifter screen. Soak and bleach the sifter as above before processing any additional batches of tomato fruit.
11. Perform antigen-specific ELISA and Western blot analysis on the bulk material to confirm antigen content and relative levels of any aggregates or higher order complexes of the protein of interest.

4. Notes

1. The tomato lines Moneymaker, Yellow Pear, Rio Grande, Micro-Tom, Great White, Medium White, and several breeding lines have been transformed using this protocol.
2. Three *Agrobacterium* strains we have used are LBA4404, GV3101, and EHA105. Although we do not see any significant difference in the number of transgenic plants recovered using these bacteria, each has advantages and disadvantages. The strain LBA4404 is our preferred strain although it is *recA* positive, and constructs must be checked regularly for recombination events. The EHA105 strain is *recA* negative and more aggressive than LBA4404. We often see EHA105 overgrowing the explants.
3. Selective agents used include: kanamycin (PhytoTechnology Laboratories), hygromycin (PhytoTechnology Laboratories), and bialaphos (PhytoTechnology Laboratories). The optimal concentration for each selective agent is dependent on the tomato variety. We have used kanamycin at 75 and 100 mg/L, hygromycin at 6 mg/L, and bialaphos at 2 and 3 mg/L. No significant difference has been seen in transformation efficiencies between experiments using the same promoters. However, we have observed differences based on selectable markers and selection agents. For instance, regeneration is slower on bialaphos than on kanamycin.
4. Substitute 500 mg/L carbenicillin if timentin cannot be acquired. We prefer timentin because it is more economical, light stable, and resistant to inactivation by β -lactamase (18), and it increases tomato shoot and root formation (19).
5. If contamination still occurs, treat the seeds with 70% ethanol for 2 min before treating with bleach.
6. Feeder layers are not essential but may increase the transformation efficiency. We have found feeder layers beneficial when the protein of interest has a negative effect on the number of shoots regenerated.
7. We have also tried hypocotyl explants; however high numbers of nontransformed shoots were recovered.

8. Using seedlings with the first true leaves dramatically reduces transformation efficiency.
9. A longer period of incubation may cause water-soaked damage to cells.
10. Lower cocultivation temperatures have increased the transformation efficiency for some crops. This has not been reported for tomato; however, we have seen increased recovery of transformed lines when expression of an introduced gene had a negative effect on transformation efficiency.
11. Plating more than 10 explants per plate reduces transformation efficiency.
12. We define transformation efficiency as percent of cotyledon explants that give rise to plants that test positive by Southern analysis. Our protocol has a tomato transformation efficiency of 10 to 14%. We find that transformation efficiency varies with different cultivars, selectable markers, and introduced genes. Some genes, when expressed, decrease the number of transformed lines recovered. An empty vector construct (no gene of interest, only the vector with a selectable marker) should be included as a transformation control to determine whether the introduced gene has such an effect.
13. We have also used PCR to determine whether plantlets are internally contaminated with *Agrobacterium* by using primers specific for genes unique to *Agrobacterium*. Plants having internal contamination should be discarded because it is not possible to rid the plants of the bacterium.
14. Washing the medium from the roots reduces the chance of adverse bacterial and fungal growth that may kill the plantlet once it is placed in soil.
15. We use plastic cups, old Magenta boxes, or clear, plastic juice containers that have the tops cut off. It is important to cover each plant immediately after transfer to soil to prevent wilting.
16. If new transfers to soil are placed in direct sunlight, heat will build up under the cover and kill the plant.
17. To acclimatize plantlets from tissue culture, we frequently mist the plantlets throughout the day. Start with intervals of 30 s every 10 min. Once hardened, the plantlets can be put on a three times daily misting cycle, 5 min per cycle. Frequency of irrigation will vary from three to five cycles per day, depending on the temperature and growth conditions. If the plants begin to wilt, another evenly spaced cycle should be added to the day.
18. Our tomatoes are grown in a BL2-P greenhouse; therefore physical barriers such as walls, doors, and insect screening prevent initial pest access. Prevention is our preferred route of pest control; therefore the older leaves are pruned off plants to eliminate insect niches, and all plants entering the greenhouse are quarantined, inspected, and, if necessary, treated before admission into the general population of healthy plants. Nevertheless, pest problems occur. Sticky yellow traps and plants are inspected on a weekly basis, or more frequently should an infestation be detected. Specifically, for control of:
 - a. White fly: apply insecticidal soap and neem oil weekly according to the manufacturer's directions. *Eretmocerus eremicus* may also be added weekly at 1.5 to 3 wasps per square meter of greenhouse space. These parasitic wasps

- should be introduced with the plants and supplemented at a 7- to 14-d intervals. The Koppert Biological Product name for *Eretmoserus eremicus* is ERCAL. If a heavy white fly infestation occurs, the Koppert product ENERMIX is more effective (*Eretmoserus eremicus* + *Encarsia formosa*).
- b. Mites: apply insecticidal soap and neem oil weekly at the recommended label rate. Repeat applications are essential for breaking the life cycle of the insect. Hexacide, a combination of natural oils, can also be used weekly. *Phytoseilus persimilis* are effective when introduced weekly at least three times, then every 3 to 4 wk. The Koppert Biological Product name for *Phytoseilus persimilis* is SPIDEX. *Mesoseilus longipes* can also be added if control is not achieved.
 - c. Fungus gnats and larvae: start with sterile potting soil and media. Discard used soil weekly. Products such as Gnatrol can be applied directly to the soil as a drench as per the manufacturer's directions. Repeat applications as needed. Use pyrethrin bombs or foggers in closed rooms with the ventilation shut off. Flying insects are killed on contact, yet pyrethrins have a short residual life. Neem oil can also be applied as a soil drench to diminish the populations over time. Repeat applications are required for effective treatment.
 - d. Thrips: remove infested plants from the growing area. Neem oil can be applied as a soil drench at the recommended label rate. Repeat application on a regular basis until the infestation is controlled. *Neoseilus cucumeris* can be used at a rate of one per 2.3 square meters of greenhouse space. The Koppert Biological Product name THRIPEX (*Amblyseius degenerans*) can be used at 5 to 100 individuals per square meter when introduced every 14 d. *Hypoaspis*, a predatory mite (ENTOMILE), can also be added at 100 to 500 per square meter on a one-time basis.
19. The time of harvest varies on the transgene expressed. We have found that the concentration of some transgenes decreased as the fruit ripened. Should this occur, the fruit should be harvested while still green.
 20. More cuts through the fruit may be required depending on the number of locules a fruit/tomato line has.
 21. We germinate kanamycin-resistant lines on $\frac{1}{2}$ MS media containing 300 mg/L kanamycin and bialaphos-resistant lines on $\frac{1}{2}$ MS media containing 3 mg/L bialaphos.
 22. If the gene of interest is following Mendelian inheritance, three out of four seeds should germinate on selection. Determining Mendelian inheritance becomes more difficult when more than one copy of the transgene is present.
 23. Because of the size of plant genomes, for Southern analysis, run the gel at least 24 h at 1 V/cm length of the gel (for a 12-cm gel, run at 12 V) to ensure good separation of high-molecular-weight DNA. Always perform two different digests on the same sample with different enzymes to ensure correct number of insertion sites and always depurinate during gel preparation for transfer to ensure transfer of high-molecular-weight DNA.

24. The volume of extraction buffer depends on the water content of the plant material to be homogenized. For ELISA analysis of fresh tomato fruit, we start with a dilution of 1 mg fruit material per 10 μ L of extraction buffer, whereas with fresh leaf material we start with a dilution of 1 mg per 14 μ L of extraction buffer.
25. To determine whether your protein of interest is being glycosylated, compare crude protein extracts with crude protein extracts that have been reacted with PNGase F (New England BioLabs, Beverly, MA) as per the manufacturer's instructions.
26. To determine whether your protein of interest is forming oligomers or polymers, compare native samples with denatured samples (protocol as given in text). During preparation of native samples, omit the DTT from the loading buffer and do not boil the sample before loading onto the gel.
27. The concentration of blocking buffer, incubation temperatures, and times presented in this protocol are a good start; however, along with antibody dilutions, these parameters require optimization. The Stratagene ECL+ detection kit gives a rudimentary optimization protocol in its instruction booklet.
28. We use Greenlight rooting hormone (Green Light Company, San Antonio, TX). Dip the end of the shoot in water, and then in the rooting hormone. Gently knock off any excess powder, and plant in soil.
29. Unless you are operating under an approved field permit for transgenic plants, all preparations should be performed within an authorized laboratory that has appropriate controls for capture of all transgenic materials such as tomato seed under biosafety level 1 or level 2 conditions. Delivery of material to any secondary facility should be documented by both parties. All equipment coming into contact with the tomatoes should be washed with a 10% bleach solution and rinsed well with water.
30. We have compared a number of processes to prepare tomato fruit for freeze drying. Processes that result in the greatest surface area with minimal cell disruption proved optimal for time required to freeze-dry and maintenance of integrity/function of the protein of interest. Pureeing decreased the time required to freeze-dry, but only 50% of the protein originally present remained active, whereas slicing the fruit resulted in 80 to 100% recovery.
31. We have successfully used a number of freeze-dryer models from large (Virtis, model 100 SRC) to small (Labconco Lyph-Lock 6 Liter, model 77530).
32. For clinical batch materials, the Date of Manufacture is defined as the date of crop planting.

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Index

A

AB* medium, preparation, 5, 9, 10

Acetosyringone, virulence gene
induction, 78, 258

Agrobacterium rhizogenes,
composite plant generation,
 Arabidopsis composite plants,
 Agrobacterium rhizogenes
 culture, 166, 167
 curing and explant recovery,
 164, 165
 hairy root selection, 165
 inoculation and coculture, 164,
 166
 seed sterilization, 162, 63, 166
materials, 157–159
non-tissue culture composite
plants,
 Agrobacterium rhizogenes
 culture, 161
 explant preparation, 159, 161,
 165
 explant recovery, 162, 165, 166
 inoculation and coculture, 161,
 162, 165
overview, 156, 157
hairy root induction, 156

Alfalfa,
 Agrobacterium-mediated
 transformation,
 Agrobacterium culture, 306, 308
 genotype identification with high
 frequency of regeneration, 305
 materials, 302–304, 308
 overview, 301, 302

plant culture,
 in vitro, 305, 308
 leaf explants, 305, 308
plant propagation, 308
somatic embryogenesis and plant
regeneration, 307, 308
transformation, 306, 307, 308
transformation approaches, 301

Arabidopsis thaliana,
composite plant generation,
 Agrobacterium rhizogenes
 culture, 166, 167
 curing and explant recovery, 164, 165
 hairy root selection, 165
 inoculation and coculture, 164, 166
 seed sterilization, 162, 63, 166

floral dip transformation,
 Agrobacterium culture, 92, 93, 99
 efficiency, 87
 insertion events, 95, 96
 materials, 89, 90, 96–98
 plant growth, 90–92, 98, 99
 principles, 87–89
 progeny testing, 95
 seed harvesting and storage, 93,
 94, 100
 selection, 94, 95, 100, 101
 single-copy insert line
 identification, 96
 transformation, 93, 99, 100
 true-breeding transgenic line
 identification, 95

root transformation,
 Agrobacterium culture, 109, 111
 applications, 106

materials, 107, 108
 overview, 105, 106
 plant growth, 109, 111
 root segment preparation and
 inoculation, 109–111
 seed sterilization, 109
 stable transformation,
 antibiotic/herbicide-resistant
 transformant scoring, 110
 crown gall tumorigenesis
 scoring, 110
 transient transformation, 110

Arachis hypogaea, see Peanut

B

Barley,

Agrobacterium-mediated
 transformation,
Agrobacterium culture, 175, 176,
 180
 efficiency, 173, 180
 embryo/callus culture and plantlet
 production, 177, 178, 181
 embryo isolation and axis
 excision, 177, 181
 infection, 177, 181
 materials, 173–175, 180
 overview, 172, 173
 plant culture, 175
 progeny screening with
 hygromycin,
 leaf assay, 180, 182
 root assay, 179, 180, 182
 pWBVec8 vector, 172, 173, 180
 seed collection and plate
 preparation, 176, 181
 transgene detection with
 polymerase chain reaction,
 178, 179, 181
 genetic engineering examples, 171, 172
 industrial uses, 171
 transformation approaches, 172

Binary vector,

Agrobacterium tumefaciens preparation
 ready for infection, 29, 31, 34, 35

components,

 accessory components, 24, 25
 selectable markers and reporters,
 19–22, 32

 T-DNA components, 19–22, 32

 unfinished plasmid stocks, 19, 31,
 32

 vector backbone components, 22,
 23, 32, 33

construction, 26, 33, 34

development, 16

examples, 26, 27

reporter gene cassette, 23, 24

types and selection, 17–19

virulence genes, 16

Brassica juncea, see Indian mustard

Brassica napus, see Canola

Brassica oleracea,

Agrobacterium-mediated
 transformation,

Agrobacterium culture, 420, 421,
 424

explant isolation, inoculation, and
 cocultivation, 421, 424

greenhouse care, 423, 424

materials, 419, 420

overview, 417–419, 424

seed sterilization and germination,
 420, 424

selection, 421, 424

shoot isolation, 421, 423–425

variants, 417

Broccoli, see *Brassica oleracea*

Brussel sprouts, see *Brassica oleracea*

C

Cabbage, see *Brassica oleracea*

Cajanus cajan, see Pigeonpea

Canola,

- Agrobacterium*-mediated transformation,
 - Agrobacterium* culture, 261, 264
 - explant preparation, 260, 261, 264
 - greenhouse care, 262, 263
 - materials, 259, 260, 264
 - overview, 257–259
 - progeny analysis,
 - polymerase chain reaction, 263
 - segregation analysis, 264
 - Western blot, 263, 264
 - transformation, 261, 262, 265
- economic importance, 258
- genetic improvement needs, 258, 259
- oil, 258
- Cauliflower, *see Brassica oleracea*
- Chickpea,
 - Agrobacterium*-mediated transformation,
 - Agrobacterium* culture, 317, 318
 - characterization of transformants, 322
 - explant preparation, 317
 - greenhouse care and seed production, 321, 322
 - infection, 318
 - materials, 314–317
 - regeneration and multiplication of shoots, 319, 320, 322
 - rooting of shoots, 320
 - shoot hardening and transplantation, 320
 - transformation approaches, 314
 - world production and growth rates, 313
- Cicer arietinum*, *see* Chickpea
- Clovers, *see also* Red clover,
 - Agrobacterium*-mediated transformation,
 - Agrobacterium* culture, 330, 331, 332
 - inoculation and cocultivation, 331, 333
 - materials, 327–330, 332
 - overview, 326, 327
 - root induction, 332, 333
 - seeds,
 - dissection, 331–333
 - sterilization, 330
 - selection and regeneration of transgenic plants, 331, 333
 - soil transfer and greenhouse care, 332
 - genetic engineering applications, 325, 326
 - transformation approaches, 326
- Composite plants,
 - Agrobacterium rhizogenes* generation, *see Agrobacterium rhizogenes*
 - examples, 156
- Cotton,
 - Agrobacterium*-mediated transformation,
 - efficiency, 269, 274
 - Agrobacterium* culture, 272, 274
 - aseptic seed germination, 272, 274
 - kanamycin resistance test, 274, 277
 - materials, 270–272
 - regeneration, embryo germination, and plantlet development, 273, 276, 277
 - selection and proliferation, 273, 275, 276
 - soil transfer, 273, 274, 277
 - transformation, 272, 274
 - genetic engineering applications, 267
 - green fluorescent protein reporter, 268, 269
 - overview, 267–270
- Cotyledon explants, *see Brassica oleracea*; *Medicago trunculata*; Soybean
- Cucumber

Agrobacterium-mediated transformation,
 green autofluorescence-emitting cell suspension transformation, 435, 437
 leaf microexplant transformation, 431, 433, 435–437
 materials, 428, 430, 431
 molecular analysis, 436, 437
 overview, 427, 428
 soil transplanting and greenhouse care, 435–437

Cucumis sativus, *see* Cucumber

Culture, *Agrobacterium*,
 AB* medium, 5, 9, 10
 antibiotics, 6, 7, 11
 complex media, 4, 5, 9, 10
 enrichment media, 6, 9, 10
 materials, 4–6, 9–11
 pH, 6, 7
 plant transformation preparations, *see* specific plants
 storage,
 desiccation, 8, 9, 11, 12
 freezing, 9, 11, 12
 overview, 3, 4
 stab culture, 8, 11
 temperature, 7

D

DNA extraction,
Agrobacterium,
 genomic DNA extraction, 70, 71, 74
 materials, 68–70, 74
 overview, 67, 68
pgl/picA locus gene integration
 confirmation, 64, 65
 plasmid isolation,
 alkaline lysis for shuttle and binary plasmids, 72, 74
 pTi or pRi plasmids, 71, 72, 74
 pea, 343, 344

peanut, 354, 356, 357
 tomato, 466, 470

E

Eggplant,
Agrobacterium-mediated transformation,
Agrobacterium culture, 443–445
 explant preparation, 443, 446
 inoculation and cocultivation, 444, 446
 maintenance of cultures and regenerated shoots, 444, 446
 materials, 440–442, 445
 overview, 440
 seed germination, 442, 445
 soil transfer and greenhouse care, 445, 446
 cultivars, 439
 genetic engineering applications, 439, 440

Electroporation,
Agrobacterium plasmids to
Escherichia coli, 72–75
 competent cell preparation, 47, 48, 50
 materials, 46, 47, 50
 parameters, 43, 44
 principles, 43
 running conditions, 48, 51

ELISA, *see* Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA), transgenic tomato analysis, 467

F

Floral dip transformation, *see Arabidopsis thaliana*
 Freeze/thaw transformation,
 competent cell preparation, 48, 49, 51
 materials, 46, 47, 50
 principles, 44

transformation conditions, 49, 51, 52

G

Gene integration, *see* *pgl/picA* locus
gene integration

Glycine max, *see* Soybean

Gossypium hirsutum, *see* Cotton

Green autofluorescence-emitting cell
suspension transformation, *see*
Cucumber

Greenhouse care, transgenic plants,

alfalfa, 308

Brassica oleracea, 423, 424

canola, 262, 263

chickpea, 321, 322

clovers, 332, 379, 382

cotton, 273, 274, 277

cucumber, 435–437

eggplant, 445, 446

Indian mustard, 286

lettuce, 454, 456

maize, 193, 194

Medicago trunculata, 124–126, 140,
142

Nicotiana, 151, 153

peanut, 354

peas, 342, 343

pigeonpea, 365, 366

rice, 210, 211

sorghum, 240

soybean, 402–404

sunflower, 296

teary bean, 412, 413

tomato, 465, 466, 469, 470

wheat, 252

H

Helianthus annuus, *see* Sunflower

Hordeum vulgare, *see* Barley

I

Indian mustard,

Agrobacterium-mediated
transformation,

Agrobacterium culture, 284

explant preparation and

inoculation, 284, 286

greenhouse acclimatization and
care, 286

materials, 282, 283, 286

overview, 281, 282

seed sterilization and germination,
284

selection and plant regeneration,
284, 286, 287

transformation approaches, 281, 282

Indica rice,

Agrobacterium-mediated
transformation,

Agrobacterium culture, 208–210

calli,

embryogenic calli

establishment, 208, 210

preculture, 209

proliferation, 209, 210

cultivar varieties, 202, 203

materials, 204–208

overview, 202

regeneration, 210, 211

selection, 209–211

soil transfer, 210, 211

transformation, 209, 210

engineering for food production,
201, 202

transformation approaches, 202, 215

J

Japonica rice,

Agrobacterium-mediated
transformation,

explant preparation, 219, 220

infection, 219, 221

materials, 216–218

overview, 215, 216

- seed sterilization, 217, 219, 220
- transformed plantlet regeneration, 219–221
- transplanting and T₁ seed production, 220, 221
- cultivar varieties, 213
- transformation approaches, 213, 215

K

Kale, *see* *Brassica oleracea*

Kohlrabi, *see* *Brassica oleracea*

L

Lactuca sativa, *see* Lettuce

Legumes, *see* Alfalfa; Chickpea;

- Clovers; *Medicago trunculata*;
- Peanut; Peas; Pigeonpea; Red clover;
- Soybean; Tepary bean

Lettuce,

- Agrobacterium*-mediated transformation,
- Agrobacterium* culture, 453, 455
- explant preparation, 453, 455, 456
- inoculation and cocultivation, 453, 456
- maintenance of cultures and regenerated shoots, 454, 456
- materials, 451, 452, 455
- overview, 450, 451
- seed germination, 453, 455
- soil transfer and greenhouse care, 454, 456
- genetic engineering applications, 450
- variants, 449

Lycopersicum esculentum, *see* Tomato

M

Maize,

- Agrobacterium*-mediated transformation,
- Agrobacterium* culture, 191, 196
- ear sterilization and embryo dissection, 191, 192, 196

- infection, cocultivation, and resting, 192, 196
- materials, 186, 188–190, 194, 195
- overview, 185–187
- plant growth for immature embryo production, 190, 191, 195, 196
- selection, 192, 196
- transgenic plants,
 - greenhouse care, 193, 194
 - regeneration, 193, 196, 197
 - seed production, 194, 197
 - transplanting and acclimation, 193, 197
- transformation approaches, 185, 186

Medicago sativa, *see* Alfalfa

Medicago trunculata,

- cotyledon explant transformation, *Agrobacterium* culture, 132
- explant preparation, 132, 134
- greenhouse care and seed harvest, 134
- inoculation and cocultivation, 132, 133
- Jemalong A17 genotype features, 130
- materials, 130, 131, 134
- seed sterilization and germination, 132
- selection and plant regeneration, 133

genome features, 129

leaf explant transformation,

- Agrobacterium* culture, 122, 125
- Basta selection, 119, 125
- callus formation, 123, 125
- cocultivation, 123, 126
- efficiency, 116
- embryogenesis, 124, 126
- leaf explant preparation and infiltration, 122, 123, 125
- materials, 116–119, 125

- overview, 115, 116
- plant material preparation,
 - greenhouse-grown plants, 121, 122, 125
 - sterilized seeds, 121, 125
- plantlet development, 124
- transgenic plant transfer to greenhouse, 124–126
- model legume system, 129, 137
- root transformation,
 - Agrobacterium* culture, 140
 - explant preparation, 140
 - greenhouse care and seed harvest, 140, 142
 - inoculation and cocultivation, 140
 - materials, 138, 139, 142
 - principles, 137, 138, 142
 - seed sterilization and germination, 139, 142
 - selection and plant regeneration, 140

N

- Nicotiana*,
 - Agrobacterium*-mediated transformation,
 - Agrobacterium* culture, 148, 149, 152
 - materials, 145–147, 151, 152
 - overview, 143–145
 - plant care and seed harvest, 151, 153
 - plant material preparation,
 - greenhouse plants, 148, 152
 - leaf explant preparation, 148
 - seeds, 148
 - progeny test and seed storage, 151
 - transformation, 149, 150, 152, 153
 - model species, 143
- Nipponbare, *see* Japonica rice

O

- Oryza sativa*, *see* Indica rice; Japonica rice

P

- PCR, *see* Polymerase chain reaction
- Peanut,
 - Agrobacterium*-mediated transformation,
 - Agrobacterium* culture, 351, 352
 - characterization,
 - DNA extraction, 354, 356, 357
 - polymerase chain reaction, 356, 357
 - culture maintenance and shoot regeneration, 352, 354, 357
 - explant preparation and cocultivation, 352, 357
 - greenhouse care, 354
 - histochemical localization of *uidA* gene activity, 354
 - materials, 348–351
 - overview, 348
 - economic importance, 347
- Peas, *see also* Chickpea; Pigeonpea,
 - Agrobacterium*-mediated transformation,
 - Agrobacterium* culture, 340, 343
 - explant preparation, 340
 - greenhouse care, 342, 343
 - inoculation, cocultivation, and selection, 340, 342, 344
 - materials, 338, 339, 343
 - overview, 337, 338
 - characterization of transformants,
 - DNA extraction, 343, 344
 - polymerase chain reaction, 343, 344
 - pgl/picA* locus gene integration,
 - confirmation of integration, DNA digestion, 64, 65
 - DNA extraction, 64, 65

eviction of starting construction, 63
 materials, 60–62, 64
 overview, 55, 56, 58, 59
 subcloning into integration vector,
 62, 64, 65
 transformation approaches, 337
 triparental mating, 62, 63, 65
 uses, 337
 vectors, 56–58
Phaseolus acutifolius, *see* Tepary bean
 Pigeonpea,
 agricultural importance, 359, 360
 Agrobacterium-mediated
 transformation,
 Agrobacterium culture, 363, 366
 characterization,
 histochemistry, 365, 366
 polymerase chain reaction,
 366
 explant preparation and culture,
 362, 363, 366
 hardening and acclimatization,
 365, 366
 materials, 360–362, 366
 overview, 360
 rooting, 365
 shoot induction, selection, and
 elongation, 363
 transplantation, 365
Pisum sativum, *see* Peas
 Plasmid extraction, *see* DNA extraction
 Polymerase chain reaction (PCR),
 barley transgene detection, 178, 179,
 181
 canola transgenic progeny analysis,
 263
 pea transformant characterization,
 343, 344
 peanut transformant characterization,
 356, 357
 pigeonpea transformant
 characterization, 366

R

Red clover,
 Agrobacterium-mediated
 transformation,
 Agrobacterium constructs and
 strain preparation, 375, 376,
 380, 381
 explant preparation, 376, 377, 381
 greenhouse care, 379, 382
 materials, 371–374, 379, 380
 overview, 370
 plant material preparation, 375, 380
 transformation, 377–379, 381, 382
 genetic engineering applications,
 369, 370
 Redgram, *see* Pigeonpea
 Rice, *see* Indica rice; Japonica rice
 RNA extraction,
 cell growth and extraction, 73–76
 materials, 68–70, 74
 overview, 67
 Root transformation, *see Arabidopsis*
 thaliana; *Medicago trunculata*
 Rye,
 Agrobacterium-mediated
 transformation,
 Agrobacterium culture, 226, 228
 cocultivation, 227, 228
 explant preparation, 226
 inoculation, 227, 228
 materials, 224–226, 228
 overview, 224
 selection, 227, 228
 transgenic plant regeneration,
 227–229
 transformation approaches, 223, 224

S

Secale cereale, *see* Rye
Solanum melongena, Eggplant
 Sorghum,
 Agrobacterium-mediated
 transformation,

- Agrobacterium* culture, 238, 239, 241
 - confirmation of transformation, 240, 241
 - efficiency, 234, 241
 - genotype assessment for transformation, 238
 - greenhouse care of transgenic plants, 240
 - immature embryo preparation, 239
 - infection and cocultivation, 239, 242
 - materials, 234–238, 241
 - overview, 233, 234
 - resting, 239
 - segregation of selectable marker from trait genes, 241, 242
 - selection, 239, 240
 - T₀ plant regeneration, 240
 - transformation approaches, 233
 - Soybean,
 - immature cotyledon explant
 - Agrobacterium*-mediated transformation,
 - Agrobacterium* culture, 401
 - explant preparation and inoculation, 401, 403, 404
 - greenhouse care, 402–404
 - materials, 398, 399, 403
 - overview, 397, 398
 - pod collection and sterilization, 401, 403
 - selection and plant regeneration, 402, 404
 - mature cotyledonary node explant
 - Agrobacterium*-mediated transformation,
 - Agrobacterium* culture, 390, 394
 - explant preparation, 391, 394
 - materials, 387–390, 394
 - overview, 386
 - seed sterilization and seedling growth, 390, 394
 - selection and plant regeneration, 391, 393–395
 - training, 386, 387
 - transplantation and greenhouse care, 393–395
 - transformation approaches, 385
 - Sunflower,
 - Agrobacterium*-mediated transformation,
 - Agrobacterium* culture, 294, 296
 - cocultivation, 294
 - explant preparation, 294
 - flowering and T₁ seed harvesting, 296
 - grafting, 295, 296
 - materials, 292, 293, 296
 - overview, 291, 292
 - seed disinfection, 294
 - selection and regeneration of transgenic plants, 294, 295, 297
 - oil, 291
 - transformation approaches, 291, 292
 - Super-binary vector,
 - Agrobacterium tumefaciens* preparation, 29, 31, 34, 35
 - components,
 - acceptor vector, 23
 - accessory components, 24, 25
 - intermediate vector, 23
 - transformation efficiency, 16, 31
 - types and selection, 17–19
 - virulence genes, 16
 - reporter gene cassette, 23, 24
 - construction and examples,
 - double T-DNA vector, 29, 34
 - single T-DNA vector, 29, 34
- T**
- Tepary bean,
 - agricultural importance, 407
 - Agrobacterium*-mediated transformation,

- Agrobacterium* culture, 411
 - callus induction, 410, 411, 413
 - cocultivation and washing, 411–413
 - greenhouse care, 412, 413
 - materials, 408–410, 413
 - overview, 408
 - seed germination, 410, 413
 - selection and regeneration, 412
 - genetic engineering applications, 407
 - Tobacco, *see* *Nicotiana*
 - Tomato,
 - agricultural importance, 459
 - Agrobacterium*-mediated transformation,
 - Agrobacterium* culture, 462
 - batch processing tomato fruit, 467, 468, 471
 - explant preparation, 463, 468, 469
 - feeder layer preparation, 463, 468
 - materials, 460–462, 468
 - overview, 460
 - plant material preparation, 463, 468
 - soil transfer and harvest, 465, 466, 469, 470
 - T₁ generation analysis,
 - DNA extraction, 466, 470
 - enzyme-linked immunosorbent assay, 467
 - protein extraction, 466
 - screening, 466, 470
 - Western blot, 466, 467, 471
 - transformation, selection, and regeneration, 463, 465, 469
 - genetic engineering applications, 459, 460
 - Transformation, *see* Electroporation; Freeze/thaw transformation; *pgl/picA* locus gene integration; specific plants
 - Trifolium* species, *see* Clovers; Red clover
 - Triparental mating,
 - cell growth and incubations, 49, 50, 52
 - materials, 46, 47, 50
 - pgl/picA* locus gene integration, 62, 63, 65
 - principles, 44, 45
 - Triticum aestivum*, *see* Wheat
- V**
- Virulence genes,
 - acetosyringone induction, 78, 258
 - induction system,
 - cell growth and harvesting, 80, 81
 - materials, 79–81
 - overview, 78, 79
 - reporter assay, 80, 81
 - super-binary vectors, 16
 - vir* regulon,
 - function, 77
 - VirA sensor, 78
 - VirG signaling, 78
- W**
- Western blot,
 - canola transgenic progeny analysis, 263, 264
 - tomato transgenic progeny analysis, 466, 467, 471
- Wheat,**
- Agrobacterium*-mediated transformation,
 - Agrobacterium* culture, 249, 252
 - callus induction, 250, 252
 - explant preparation, 248, 249, 252
 - inoculation and cocultivation, 249, 250, 252
 - materials, 246–248, 252
 - overview, 245, 246
 - plant regeneration, 250, 252
 - selection, 250, 252
 - soil transfer, 252
 - stock plant growth, 248
 - genetic engineering for food production, 245
- Z**
- Zea mays*, *see* Maize