31

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

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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 ddH_2O.
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 ddH_2O, and 100 µL Tween-20 (polyoxyethylenesorbitan monolaurate, e.g., Sigma P-7949).
3. Sterile ddH_2O: 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-µm 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-napthaleneacetic 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 ddH_2O, 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_2O	Filter
Spectinomycin	1000X	100	ddH_2O	Filter
Rifampicin	1000X	25	DMSO	None
Timentin	500X	250	ddH_2O	Filter

[a]*See* **Note 6**.

Table 2
Hormone Stock Solutions

Hormone	Concentration (mg/mL)	Amount (mg)	Solvent	Diluent
NAA	2.00	100	800 µL 1 *N* NaOH	ddH_2O to 50 mL
Kinetin	2.12	53	400 µL 1 *N* NaOH	ddH_2O to 25 mL
2,4-D	1.00	50	5 mL Ethanol	ddH_2O to 50 mL
Adenine	2.00	50	—	ddH_2O 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 ddH_2O; 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 × 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 ddH_2O; 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 ddH_2O 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

[a] *See* **Note 9**.

[b] *See* **Note 4**.

[c] Many, 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).

[d] *See* **Note 5**.

media, sprinkle Gamborg's B5 basal salts over approximately 900 mL ddH_2O 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 ddH_2O. 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-cm^2 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 µmol/m^2/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 µmol/m^2/s illumination from fluorescent lamps until established. Plants may be transferred to the greenhouse with appropriate acclimation to the higher (i.e., 400–1000 µmol/m^2/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, DH5α, 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 µL *Agrobacterium* culture with 300 µL 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 (OD_{600nm} 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_2O 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_2O 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 ddH_2O 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 µmol/m^2/s illumination from fluorescent lamps (*see* **Note 20**).

1. Transfer harvested, surface-sterilized petioles (*see* **Subheading 3.3.** above) to a sterile 15 × 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 × 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 µmol/m^2/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 µmol/m^2/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 *nptII* 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 µL H_2O 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 ddH_2O 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 ddH_2O 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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References

1. Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W. (1987) Gus fusions—β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6,** 3901–3907.
2. Sullivan, M. L., Hatfield, R. D., Thoma, S. L., and Samac, D. A. (2004) Cloning and characterization of red clover polyphenol oxidase cDNAs and expression of active protein in *Escherichia coli* and transgenic alfalfa. *Plant Physiol.* **136,** 3234–3244.
3. Quesenberry, K. H., Wofford, D. S., Smith, R. L., Krottje, P. A., and Tcacenco, F. (1996) Production of red clover transgenic for neomycin phosphotransferase II using *Agrobacterium*. *Crop Sci.* **36,** 1045–1048.
4. Wofford, D. S., Baltensperger, D. D., and Quesenberry, K. H. (1992) *In vitro* culture responses of alyceclover genotypes on four media systems. *Crop Sci.* **32,** 261–265.
5. Wan, Y., Sorensen, E. L., and Liang, G. H. (1988) Genetic control of *in vitro* regeneration in alfalfa (*Medicago sativa* L). *Euphytica* **39,** 3–9.
6. Quesenberry, K. H. and Smith, R. R. (1993) Recurrent selection for plant regeneration from red clover tissue culture. *Crop Sci.* **33,** 585–589.
7. Smith, R. R. and Quesenberry, K. H. (1995) Registration of NewRC red clover germplasm. *Crop Sci.* **35,** 295–295.
8. Gleave, A. P. (1992) A versatile binary vector system with a T-DNA organizational-structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Mol. Biol.* **20,** 1203–1207.
9. Rogers, S. G., Klee, H. J., Horsch, R. B., and Fraley, R. T. (1987) Improved vectors for plant transformation—expression cassette vectors and new selectable markers. *Methods Enzymol.* **153,** 253–277.
10. Hellens, R., Mullineaux, P., and Klee, H. (2000) A guide to *Agrobacterium* binary Ti vectors. *Trends Plant Sci.* **5,** 446–451.
11. Gamborg, O. L., Miller, R. A., and Ojima, K. (1968) Nutrient requirement of suspension cultures of soybean root cells. *Exp. Cell Res.* **50,** 151–158.

12. Moore, G. A., Jacono, C. C., Neidigh, J. L., Lawrence, S. D., and Cline, K. (1992) *Agrobacterium*-mediated transformation of citrus stem segments and regeneration of transgenic plants. *Plant Cell Rep.* **11,** 238–242.
13. Rogers, S. G., Horsch, R. B., and Fraley, R. T. (1986) Gene transfer in plants—production of transformed plants using Ti plasmid vectors. *Methods Enzymol.* **118,** 627–640.
14. Taylor, N. L. (1980) Red clover, in *Hybridization of Crop Plants* (Fehr, W. R. and Hadley, H. H., eds.), American Society of Agronomy and Crop Science Society of America, Madison, WI, pp. 261–272.
15. Adair, D., Irwin, R., and Traynor, P. L. (2001) *A Practical Guide to Containment*. Information Systems for Biotechnology, Blacksburg, VA.