25

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)***.

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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 Hildebrant basal salt mixture (Sigma), 1 mL/L 1000X Schenk and Hildebrant 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 SHII micronutrient stock A, 1 mL/L SHII micronutrient stock B, 5 mL/L SHII iron stock, 10 mL/L SHII 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 $CaCl_2 \cdot 2\ H_2O$, 25 g/L KNO_3, 4 g/L $MgSO_4 \cdot 7\ H_2O$, 3 g/L $NH_4H_2PO_4$.
   b. SHII micronutrient stock A: 5 g/L boric acid, 0.1 g/L $CoCl_2 \cdot 6\ H_2O$, 0.2 g/L $CuSO_4 \cdot 5\ H_2O$, 10 g/L $MnSO_4 \cdot H_2O$, 1 g/L $ZnSO_4 \cdot 7\ H_2O$.
   c. SHII micronutrient stock B: 1.0 g/L KI, 0.1 g/L $Na_2MoO_4 \cdot 2\ H_2O$.
   d. SHII iron stock: 3 g/L $FeSO_4 \cdot 7\ H_2O$ and 4 g/L Na_2EDTA dissolved in hot distilled water.
   e. SHII 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 KNO_3, 0.25 g/L $MgSO_4 \cdot 7\ H_2O$, 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 Phytablend (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 Phytablend (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 Phytablend. 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 μE/m^2/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 × 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 μE/m^2/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 μE/m^2/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 μE/m^2/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 μE/m^2/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 trifoliolate 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 A_{600} 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 $\mu E/m^2/s$ (place a piece of cheesecloth over plates in a chamber at 60–80 $\mu E/m^2/s$) 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 μE/m²/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 μE/m²/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 μE/m²/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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