**Agrobacterium tumefaciens–Mediated Transformation of Three Milkweed Species (Asclepias hallii, A. syriaca, and A. tuberosa: Apocynaceae)**

Patricia Keen,¹ Amy Picard Hastings,² Anurag A. Agrawal,² and Joyce Van Eck¹,3,4

¹The Boyce Thompson Institute, Ithaca, New York
²Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, New York
³Plant Breeding and Genetics Section, School of Integrative Plant Science, Cornell University, Ithaca, New York
⁴Corresponding author: jv27@cornell.edu

Milkweeds have ecological significance for insect herbivores that rely on them as hosts for either part of or the entirety of their life cycles. Interesting interactions, some of which are not completely understood, have evolved over time. To develop these species as models to elucidate the interplay with insect herbivores, we established Agrobacterium tumefaciens–mediated transformation approaches for Asclepias hallii (Hall’s milkweed), A. syriaca (common milkweed), and A. tuberosa (butterflyweed). The method is based on infection of stem internodal explants, which were more amenable to transformation than leaf explants. We found that addition of freshly prepared dithiothreitol was critical to prevent browning of stem explants. Depending on the species, the time from infection to the regeneration of transgenic lines ranges from 2 to 4 months. Transformation efficiency for A. hallii was 9%, whereas efficiencies for A. syriaca and A. tuberosa were 6% and 13%, respectively. © 2020 by John Wiley & Sons, Inc.

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**INTRODUCTION**

The plant genus Asclepias contains more than 140 species that are commonly referred to as milkweeds because they produce white, sticky latex (Fishbein et al., 2018; Woodson, 1954). The latex and most other plant tissues contain cardenolides (steroidal toxins), which are inhibitors of animal sodium-potassium ATPases that provide defense against insects (Agrawal, Petschenka, Bingham, Weber, & Rasmann, 2012). Due to milkweed’s potent defenses, it is host to very few, highly specialized insect herbivores, including
the monarch butterfly (*Danaus plexippus*). As an ecological model system for studying plant–insect interactions, milkweed is well suited for investigations of regulation and deployment of defenses. Thus, our long-term interest is to develop *Asclepias* as a model to support gene function studies related to characteristics such as defense signaling, production of cardenolides and latex, and ecological interactions between plants and insect herbivores including the monarch butterfly.

A key requirement for adoption of a plant species as a model for research is availability of gene transfer (transformation) methods. In this article, we describe a method that has been successful for the recovery of transgenic lines from three different *Asclepias* species native to the United States (*A. hallii*, *A. syriaca*, *A. tuberosa*). The method is based on *Agrobacterium tumefaciens* infection of stem internode explants from plants grown in vitro. We found that stem internode explants resulted in more efficient transformation than leaf explants. We provide details on establishment of plant material both in soil and in vitro as sources of explants, infection of internode explants, recovery of transgenic lines, and approaches for rooting these lines ex vitro. To our knowledge, this is the first report of an approach to recover transgenic milkweed.

**BASIC PROTOCOL 1**

*Agrobacterium tumefaciens–MEDIATED TRANSFORMATION OF Asclepias INTERNODAL STEM EXPLANTS*

In this protocol, we describe the method for transformation of three different *Asclepias* species (*A. hallii*, *A. syriaca*, *A. tuberosa*). The method is based on *Agrobacterium tumefaciens* infection of stem internode explants from seedling material; however, in vitro propagated plants could be established for longer-term efforts where a continued source of material would be required. An established in vitro population also provides contaminant-free material as compared with seedling material.

The protocol is composed of five stages: (1) seed disinfection, seedling growth, and establishment of in vitro plants; (2) *Agrobacterium* culture preparation; (3) *Agrobacterium*-mediated transformation of internode explants; (4) plant regeneration; and (5) rooting of transgenic lines ex vitro.

**Materials**

- *A. hallii*, *A. syriaca*, and *A. tuberosum* seeds
- 10% and 20% (v/v) bleach
- Household dish soap
- Metro-Mix 360 soilless potting mix (e.g., Sun Gro Horticulture)
- Jack’s Professional Fertilizer, LX 21-5-20 (e.g., JR Peters)
- *Agrobacterium tumefaciens* strain (LBA4404) harboring construct (see Basic Protocol 2)
- LB plates with appropriate selection agent (see recipe)
- YEP liquid medium containing appropriate selection agents (see recipe)
- Pulse preculture medium (see recipe)
- TAJ preculture medium (see recipe)
- 70% (v/v) ethanol
- Tween 20
- 100 mM dithiothreitol (DTT; e.g., PhytoTechnology Laboratories, cat. no. D259)
- 1 mg/ml 2,4-dichlorophenoxyacetic acid (2,4-D; e.g., PhytoTechnology Laboratories, cat. no. D295)
- 1 mg/ml 6-benzylaminopurine (BAP; e.g., PhytoTechnology Laboratories, cat. no. B130)
- Pulse liquid medium (see recipe)
- 1 mg/ml trans-zeatin (see recipe)
- 1 mg/ml 1-napthaleneacetic acid (NAA; see recipe)
TAJ liquid medium (see recipe)
Pulse selective medium (see recipe)
CIM selective medium (see recipe)
Post pulse selective medium (see recipe)
Plant development selective medium (see recipe)
Elongation selective medium (ESM; see recipe)
Liquid nitrogen
Acetyltrimethylammonium bromide (CTAB) buffer (e.g., Promega) containing 0.2% 2-mercaptoethanol
Chloroform
Isopropanol
10% Tris-EDTA
Hormex 8 (e.g., Hummert International)
Clonex rooting gel (e.g., Hydrodynamic International)
Clonex Clone Solution (e.g., Hydrodynamics International)
Scalpel with no. 10 blade (e.g., Feather Safety Razor)
Paper towel or filter paper
60 × 15–mm, 100 × 15–mm, and 100 × 20–mm untreated plastic petri dishes, sterile (e.g., Corning)
Parafilm
Variable temperature incubators, 4°C to 30°C
10-cm plastic plant pots
28 × 61–cm plant growth trays
5- and 15-cm tall humidity domes
Plant grown chambers with the following conditions:
  24°C day, 22°C night, 16-hr photoperiod, 60 to 100 μmol/m²/s light
  27°C day, 24°C night, 14-hr photoperiod, 350 μmol/m²/s light
  30°C day and night, dark
Forceps
Spectrophotometer
50-ml centrifuge tube
Shaking incubator
Centrifuge (e.g., Sorvall, SS34 rotor)
12-well plate
Vacuum filtration apparatus for infiltration
Micropore tape, 1.25-cm medical sealing tape (e.g., Micropore)
Magenta GA-7 vessels and lids (e.g., Sigma-Aldrich)
1.5-ml microcentrifuge tubes
Mixer Mill (e.g., Retsch, MM 300)
3-mm stainless steel beads
Microcentrifuge
Microvolume spectrophotometer (e.g., NanoDrop)
Heat mat
Hydroponic (root misting) chamber (e.g., EZ Clone Enterprises)
Fog tunnel (white plastic sheets with fogger)
Neoprene

Additional reagents and equipment for PCR (see Current Protocols article: Kramer & Coen, 2001)

**Germinate seed and grow seedling (all three milkweed species)**

1. Surface-sterilize seeds by soaking in 30 ml of 20% (v/v) bleach in deionized water containing one drop of dish soap for 15 to 20 min with periodic shaking. Rinse seeds thoroughly with deionized water.
2. Scarify seeds by shaving gently with a scalpel at the tapered end of the seed (where the radicle will emerge) and along the top ridge in the seed coat to expose a small amount of white tissue.

3. Place seeds between layers of paper towel or filter paper in a petri dish. Moisten layers with water, and seal petri dishes with Parafilm.

4. Stratify seeds for 7 days at 4°C, and then move to a 30°C chamber. Keep seeds in the dark.

   *Seeds will typically germinate in 3 to 5 days (Fig. 1).*

5. Transfer seedlings into 10-cm greenhouse pots prefilled with moistened Metro-Mix 360 soilless mix, such that the radicle and seed coat are in the soil and the hypocotyl hook is exposed to light. Place pots in growth trays, and cover seedlings with a 5-cm humidity dome. Place in a growth chamber with 350 μmol/m²/s light, 14-hr photoperiod, and daytime temperature of 27°C (night temperature, 24°C). Water as needed.

6. Remove humidity dome as cotyledons emerge from seed coats. If needed, mist with water, and use forceps to loosen seed coats to promote germination. Apply one dose of Jack’s Professional LX 21-5-20 fertilizer applied as a single 150 ppm dose at first dry-down. Grow plants for 2 weeks. Water as needed, and keep free from pests.
Prepare Agrobacterium cultures (days 1, 2, and 3)

7. Day 1: Streak *Agrobacterium* from a glycerol stock onto LB plates containing the appropriate selection agents. Incubate at 28°C for 2 days.

8. Day 3: Select 4 well-formed colonies from the LB plates, and transfer to 50 ml YEP liquid medium containing appropriate selection agents. Culture in a shaking incubator at 250 rpm at 2°C overnight or longer to reach an optical density at 600 nm (OD$_{600}$) of 0.6.

Prepare explants (day 3)

9. For *A. hallii* and *A. tuberosa*, prepare explants from stems of 3-week-old and 4- to 8-week-old seedlings, respectively. Make cuts through the seedling stem immediately above cotyledons and remove leaves. Transfer explants from up to 8 seedlings to a 50-ml centrifuge tube containing 30 ml water and a few drops of household dish soap.

10. Agitate on a rotary shaker at 200 rpm for 20 min.

11. Rinse material three times in deionized water. Drain last rinse from centrifuge tube, and fill with 70% ethanol. Soak for 1 min.

12. In a laminar flow hood, decant ethanol and add 30 ml of 10% bleach containing one drop of Tween 20. Agitate on a rotary shaker at 200 rpm for 20 min. Rinse three times in sterile, deionized water, leaving material in water until cutting (step 13).

13. Cut 0.5- to 1.5-cm internodal stem pieces. Place on pulse preculture medium for 1 day. Preculture in the dark at 24°C ± 2°C. Wrap plate with Parafilm.

*For A. syriaca, we established a population of in vitro plants because of a larger, long-term effort with this species. The methods described for A. syriaca could also be applied to other milkweed species.*

*In vitro plants were established from nodal explants from 2- to 2.5-week-old seedlings. Propagate nodal cuttings on post pulse medium in test tubes or Magenta boxes. Recut nodal explants from the in vitro population, and transfer to freshly prepared post pulse medium every 4 to 6 weeks. For transformation, cut 0.5- to 1-cm internodal explants from 8- to 10-cm tall plants. For A. syriaca, preculture for 1 day on TAJ preculture medium. Wrap plate with Parafilm. Maintain cultures in the dark at 24°C ± 2°C.*

Inoculate explants (day 4)

14. Check OD$_{600}$ of *Agrobacterium* liquid culture (optimally 0.6 to 0.7). Centrifuge 10 min at ~8000 × g, 20°C.

15. Prepare inoculation solution: For *A. tuberosa* and *A. hallii*, add 125 μl of 100 mM DTT, 50 μl of 1 mg/ml 2,4-D, and 5 μl of 1 mg/ml BAP to 50 ml pulse liquid medium. For *A. syriaca*, add 100 μl of 1 mg/ml trans-zeatin, 12.5 μl of 1 mg/ml NAA, and 125 μl of 100 mM DTT to 50 ml TAJ liquid medium. Gently mix by inverting the tube five times. Prepare a second 50-ml tube of TAJ liquid medium for the second dilution containing the same additions.

*DTT should be prepared fresh before use and filter sterilized using a 0.22-μm filter.*

16. Discard supernatant (from step 14), and bring to 50 ml with the inoculation solution prepared in step 15. For *A. syriaca*, further dilute *Agrobacterium* to 1 × 10$^{-3}$ by pipetting 50 μl *Agrobacterium* solution into 50 ml TAJ liquid medium.

17. For *A. hallii* and *A. tuberosa*, transfer 12 to 13 internodal stem segments to one well of a 12-well plate containing 3 ml inoculation solution. Repeat for the other plates. Incubate for 10 min, agitating occasionally.
18. Transfer 80 internodal stem pieces of *A. syriaca* to a 60 × 15–mm plate containing 10 ml of the appropriate inoculation solution. Vacuum infiltrate for 30 min, agitating occasionally. Gradually release vacuum. For postinfiltration recovery, transfer plates to a rotary shaker for 30 min at 50 to 60 rpm.

19. Briefly blot all explants from all species on sterile paper towels to remove excess inoculation solution. Return explants to the original plates of culture medium. Wrap plates with Parafilm. Maintain *A. hallii* and *A. tuberosa* for 2 days and *A. syriaca* for 7 days in the dark at 22°C.

**Regenerate plant**

20. After cocultivation, transfer 15 to 20 *A. hallii* and *A. tuberosa* explants to each plate of pulse selective medium. Transfer the same number of *A. syriaca* internodal pieces to each plate of CIM selective medium. Transfer 10 control explants (not inoculated with *Agrobacterium*) of each species to one plate each of their respective selective and nonselective media. Seal plates with micropore tape, and maintain in dark conditions at 24°C ± 2°C. Frequently assess cultures for contamination.

21. Transfer all cultures to freshly prepared medium every 2 weeks.

22. At 4 weeks after inoculation, transfer *A. tuberosa* and *A. hallii* cultures to light conditions (16 hr light, 8 hr dark photoperiod; 57 to 65 μE/m²/s) at 24°C ± 2°C. Transfer explants to post pulse selective medium when callus is observed. For *A. syriaca*, when white-colored callus begins to form, transfer callus to plant development selective medium. Transfer callus with shoots to light conditions (16 hr light, 8 hr dark photoperiod; 57 to 65 μE/m²/s) at 24°C ± 2°C.

*Shoots should begin to appear 1 to 4 weeks after being transferred off the auxin. Shoots begin to regenerate 2 to 4 months after transformation (Fig. 2A,B).*

23. Transfer shoots of *A. hallii* and *A. tuberosa* to ESM in Magenta GA7 vessels when shoots are 1 cm tall (Fig. 2C). Transfer *A. syriaca* shoots to plant development selective medium in Magenta GA7 vessels.

24. Use PCR analysis to verify lines are transgenic. For DNA isolation collect one small leaf piece (1 to 2 cm²), place in a 1.5-ml microcentrifuge tube, and place on dry ice. Store at −80°C until extraction.
Asclepias syriaca rooted according to the soil method. 

(A) Transgenic shoots on plant development selective medium. (B) Transgenic lines ~2 months after transfer to soil.

25. For extraction, grind tissue (on liquid nitrogen) into a fine powder using a Mixer Mill and 3-mm stainless steel beads. Incubate samples at 60°C in CTAB buffer containing 0.2% 2-mercaptoethanol for 20 min. Centrifuge to remove particulates, and extract lysate with chloroform.

26. Precipitate DNA from the aqueous phase with isopropanol. Wash with 70% ethanol, and dry and resuspend DNA in 10% Tris-EDTA. Assess quantity and quality with a microvolume spectrophotometer. Dilute samples with water to yield approximate DNA concentrations of 5 to 20 ng/μl.

**Transfer to soil and acclimatize**

We have extensively investigated rooting methods for A. syriaca but not the other two species. We have used two different matrices for rooting: soil and a hydroponic root misting chamber (Clonex). We present both methods depending on access to a hydroponic chamber. We believe that both approaches will also be applicable for A. hallii and A. tuberosa.

27. For both rooting methods: Transfer in vitro shoots to soil when they are ~4 to 6 cm tall (Fig. 3A). Remove lower leaves if needed, and make a 45° angle cut at the base of the stem with a scalpel or razor blade. Immediately place in tempered water to avoid air bubbles from forming in the stem. Gently wash residual culture medium from the stem base. Dip cut area in Hormex 8 (soil method) or rooting gel (Clonex method; Fig. 4A).

28. Soil method: Perform the soil method as follows:

   a. Transfer shoots to moistened soil (Metro-Mix) in plant growth trays (28 × 61 cm), and cover with a 15-cm humidity dome.
   b. Place on a heat mat in a growth chamber with 14 hr day length and lighting and temperatures similar to conditions in the tissue culture facility. Monitor flats for moisture, bottom watering as needed and misting daily for the first week.
   c. Gradually begin to vent the dome slightly during the day as plants grow, and adjust to lower humidity.
   d. After 7 to 10 days, move flat along with its dome directly onto a heat mat on a greenhouse bench shaded by shade cloth. Continue to monitor plants for ~2 months, bottom watering, misting, and venting as needed.
After ∼2 weeks, begin to bottom water cuttings in a dilute fertilizer solution. Without nutrients, the plants will typically begin to yellow around 2 to 3 weeks. Plants can also be misted with one-quarter strength Jack’s Professional fertilizer during this time. Look for plants to grow new leaves and for roots to appear after ∼2 months (Fig. 3B). Seasonality seems to play a role in successful rooting in the greenhouse; success rates have been highest in the late fall in upstate New York, with the highest success at ∼40%. Rooting rates have otherwise typically been lower than 10%.

29. **Clonex method**: Perform the Clonex method as follows:

a. Prepare a hydroponic root misting chamber, such as the EZ Clone (Fig. 4B). Fill with a fresh solution of Clonex Clone Solution (5 ml/L) in tempered water, and adjust pH to 5.5 to 6.5.

b. Place in a fog tunnel (tunnel made from white plastic sheeting with a fogger for increased humidity) in a greenhouse.

c. Place freshly cut, dipped cuttings into neoprene collars with at least 1 in. of stem hanging below the collar.

d. Plug in machine, and set to constantly mist roots.

e. Change clone solution and adjust pH weekly.

f. Once plants have multiple, branched roots >1-in. long, plant in well-drained soil (Fig. 4C).

30. Gradually transition out of high-humidity environment by covering with a vented dome or plastic bag and increasing venting over the course of 1 week. Gradually transition rooted cuttings out of the shade.

**PREPARATION OF Agrobacterium GLYCEROL STOCKS CONTAINING GENE CONSTRUCTS**

Here we describe preparation of glycerol stocks following the introduction of binary vector constructs into *Agrobacterium tumefaciens*. Large supplies of electrocompetent *Agrobacterium tumefaciens* LBA4404 are prepared and maintained at −80°C as a source of cells for electroporation to introduce plasmid DNA. Colony PCR is used to verify the presence of the vector in selected colonies following electroporation. The process has five stages: (1) generation of electrocompetent cells; (2) electroporation; (3) culture on selective solidified culture medium; (4) colony PCR analysis; and (5) glycerol stock preparation.

**Materials**

*Agrobacterium tumefaciens* strain LBA4404  
LB solid medium containing rifampicin (see recipe)  
YENB liquid medium (see recipe)
10% and 50% (v/v) glycerol, sterile (e.g., Fisher Scientific, cat. no. BP-229)

Binary plasmid construct containing selectable marker cassette and gene of interest

S.O.C. medium (e.g., Invitrogen, cat. no. 46-0700)

2× Go Taq Green Master Mix (e.g., Promega, cat. no. M712B)

10 μM forward primer suitable for gene of interest

10 μM reverse primer suitable for gene of interest

YEP liquid medium (see recipe)

28°C shaking incubator

Spectrophotometer (e.g., Eppendorf BioPhotometer)

Centrifuge

50-ml centrifuge tubes, sterile

Electroporation cuvettes

0.6- and 1.7-ml microcentrifuge tubes, sterile (e.g., LPS, cat. nos. L211151 and L211511, respectively)

Electroporator (e.g., Eppendorf Model 2510)

PCR tubes

Thermal cycler

Cryovials (e.g., Fisher Scientific, cat. no. 03-337-7X)

Prepare competent Agrobacterium for electrotransformation

1. Streak Agrobacterium LBA4404 on LB medium containing rifampicin, and incubate at 28°C for 2 to 3 days.

2. Inoculate a single Agrobacterium colony in 5 to 10 ml YENB liquid medium, and grow overnight at 28°C in a shaking incubator at 250 rpm.

3. Inoculate 500 ml YENB liquid medium with 2.5 to 5.0 ml fresh overnight culture, and grow cells at 28°C in a shaking incubator at 250 rpm. Harvest cells when cultures reach OD<sub>600</sub> of 0.5 to 0.9. If cells are overgrown, dilute back to an OD<sub>600</sub> of ~0.2, and regrow to desired OD<sub>600</sub>.

4. Chill culture on ice for 5 min. Centrifuge 10 min at 4000 × g, 4°C. Remove and discard medium. Keep pelleted cells on ice. Resuspend in 5 ml sterile cold water. Transfer to 50-ml centrifuge tubes. Fill to top with sterile water. Repeat centrifugation and resuspension.

5. Resuspend in 20 ml cold 10% glycerol. Centrifuge 10 min at 4000 × g, 4°C. Remove and discard supernatant.

6. Resuspend cells in a final volume of 1 to 1.5 ml cold sterile 10% glycerol. Prepare 50-μl aliquots, and keep frozen at −80°C until needed.

Perform electrotransformation of electrocompetent Agrobacterium

7. Remove volume of Agrobacterium LBA4404 needed from the −80°C freezer, and immediately put on ice.

   We use 50 μl electrocompetent Agrobacterium for each construct (plasmid) of interest.

8. Place cuvettes and 0.6-ml microcentrifuge tubes for mixing Agrobacterium with plasmid on ice. Transfer 50 μl Agrobacterium to a cold 0.6-ml microcentrifuge tube. Add 1.5 μl DNA. Gently resuspend by pipetting up and down.

9. Transfer resuspended DNA to chilled cuvettes.

10. Perform electroporation, following the manufacturer’s instructions for your instrument.

11. Immediately add 200 μl S.O.C. medium to cuvette after electroporation.
12. Mix well by pipetting up and down. Transfer solution to a 1.7-ml microcentrifuge tube.

13. Incubate tubes 1.5 hr in a shaking incubator at 28°C.

14. Transfer entire content of tube onto selective LB medium, and distribute according to your laboratory’s method.

15. Incubate culture at 28°C for 2 days.

**Perform colony PCR**

16. Lightly touch five colonies with individual sterile toothpicks, and drop each into a PCR tube containing a master mix solution with the following components (20 μl total volume):

   - 10 μl of 2× Go Taq Green Master Mix
   - 0.3 μl of 10 μM forward primer
   - 0.3 μl of 10 μM reverse primer
   - 7.4 μl sterile water

17. Perform PCR using the thermal cycler program that best suits your primer selections.

**Prepare glycerol stocks**

18. Based on PCR results, select a colony of interest, and inoculate 2 ml YEP liquid medium containing selective antibiotic(s). Incubate at 28°C in a shaking incubator at 250 rpm for 16 to 18 hr or until OD$_{600}$ reaches 1.0. If OD$_{600}$ is more than 1.0, dilute and regrow to within 0.05 of the target OD$_{600}$.

19. Add 300 μl of 50% (v/v) glycerol to a cryovial.

20. Add 600 μl Agrobacterium culture to the glycerol. Gently mix by pipetting up and down five times.

21. Cap and store at −80°C.

**REAGENTS AND SOLUTIONS**

**3R vitamins**

- 100 mg thiamine HCl (e.g., PhytoTechnology Laboratories, cat. no. T390)
- 50 mg nicotinic acid (e.g., PhytoTechnology Laboratories, cat. no. N765)
- 50 mg pyridoxine HCl (e.g., PhytoTechnology Laboratories, cat. no. P866)
- Bring to 100 ml with deionized water
- Store 1-ml aliquots at 4°C for up to 6 months

**CIM selective medium**

- 4.3 g Murashige and Skoog (MS) salts (e.g., Caisson, cat. no. MSP01)
- 1 ml 3R vitamins (see recipe)
- 100 mg myo-inositol
- 30 g sucrose
- Adjust pH to 5.9 with HCl or KOH as needed
- 8 g agar (e.g., Sigma-Aldrich, cat. no. A1208)
- Bring to 1 L with deionized water
- Sterilize by autoclaving and cool to 55°C to 60°C in a water bath
- Supplement with 0.25 ml of 1 mg/ml NAA (see recipe), 6 ml of 1 mg/ml trans-zeatin ribose (see recipe), 2.5 ml of 100 mM DTT, 1 ml of 100 mg/ml kanamycin, and 3 ml of 100 mg/ml timentin
- Store at 4°C for up to 1 month or at room temperature for up to 2 weeks

*NAA, trans-zeatin ribose, DTT, kanamycin, and timentin solutions should be filter sterilized using a 0.22-μm filter before use.*
**ESM**

- 4.3 g MS salts (e.g., Caisson, cat. no. MSP01)
- 1 ml Nitsch vitamins (see recipe)
- 30 g sucrose
- Adjust pH to 6.0 with KOH
- 8 g Bacto agar (e.g., BD, cat. no. 214010)
- Bring to 1 L with deionized water
- Supplement with 0.75 ml of 100 mg/ml kanamycin, 2.5 ml of 100 mM DTT, and 3 ml of 100 mg/ml timentin
- Store at 4°C for up to 1 month or at room temperature for up to 2 weeks

*Kanamycin, DTT, and timentin solutions should be filter sterilized using a 0.22-μm filter before use.*

**LB medium**

- 10 g/L tryptone (e.g., BD, cat. no. 211705)
- 5 g/L yeast extract (e.g., BD, cat. no. 212750)
- 10 g/L NaCl (e.g., Fisher, cat. no. BP358)
- 15 g/L Bacto agar (e.g., BD, cat. no. 214010)
- Sterilize by autoclaving and cool to 55°C to 60°C in a water bath
- Supplement with appropriate antibiotic selection agents before pouring into plates
- Store at 4°C for up to 1 month

**LB solid medium containing rifampicin**

- 10 g/L tryptone (e.g., BD, cat. no. 211705)
- 5 g/L yeast extract (e.g., BD, cat. no. 212750)
- 10 g/L NaCl (e.g., Fisher, cat. no. BP358)
- 15 g/L Bacto agar (e.g., BD, cat. no. 214010)
- Sterilize by autoclaving and cool to 55°C to 60°C in a water bath
- Supplement with 1.25 ml of 12 mg/ml rifampicin
- Pour into sterile 100 × 15–mm plates (24.5 ml/plate)
- Store at 4°C for up to 1 month

**NAA, 1 mg/ml**

- 10 mg NAA (e.g., Caisson, cat. no. N001)
- Dissolve with a few drops of 1 M KOH
- Bring to 10 ml with water
- Filter sterilize using a 0.22-μm filter
- Store at 4°C for up to 6 months

**Nitsch vitamins, modified, 1000×**

- 0.1 g glycine
- 0.5 g nicotinic acid
- 0.025 g pyridoxine HCl
- 0.025 g thiamine HCl
- 0.025 g folic acid
- 0.002 g D-biotin
- Bring to 40 ml with water
- Slowly bring pH to 7.0 with HCl or KOH as needed
- Store 1-ml aliquots at 4°C for up to 6 months

*The solution will go from cloudy to clear as the pH approaches 7.0.*

**Plant development selective medium**

- 4.3 g MS salts (e.g., Caisson, cat. no. MSP01)
- 1 ml 3R vitamins (see recipe)
100 mg myo-inositol
30 g sucrose
Adjust pH to 5.9 with KOH
8 g agar (e.g., Sigma-Aldrich, cat. no. A1208)
Bring to 1 L with deionized water
Sterilize by autoclaving and cool to 55°C to 60°C in a water bath
Supplement with 6 ml of 1 mg/ml trans-zeatin riboside (see recipe), 1 ml of
100 mg/ml kanamycin, 3 ml of 100 mg/ml timentin, and 2.5 ml of 100 mM DTT
Store at 4°C for up to 1 month or at room temperature for up to 2 weeks
Trans-zeatin ribose, kanamycin, timentin, and DTT solutions should be filter sterilized using
a 0.22-μm filter before use.

Do not add kanamycin to medium used for positive control plants.

**Post pulse selective medium**

4.3 g MS salts (e.g., Caisson, cat. no. MSP01)
0.4 ml of 1 mg/ml thiamine HCl
2 ml of 1 mg/ml glycine
100 mg myo-inositol
1 ml of 0.5 mg/ml pyridoxine HCl
1 ml of 0.5 mg/ml nicotinic acid
30 g sucrose
Adjust pH to 5.7 with KOH
7 g agar (e.g., Sigma-Aldrich, cat. no. A1208)
Bring to 1 L with deionized water
Sterilize by autoclaving and cool to 55°C to 60°C in a water bath
Supplement with 4 ml of 1 mg/ml BAP, 1.5 ml of 1 mg/ml NAA (see recipe),
0.75 ml of 100 mg/ml kanamycin, 2.5 ml of 100 mM DTT, and 3 ml of 100 mg/ml timentin
Store at 4°C for up to 1 month or at room temperature for up to 2 weeks
Thiamine HCl is light sensitive; it should be wrapped in foil and stored at 4°C for up to 6 months. Glycine, pyridoxine HCl, and nicotinic acid solutions should be stored as 1-ml aliquots at −20°C for up to 6 months.
BAP, NAA, kanamycin, DTT, and timentin solutions should be filter sterilized using a 0.22-μm filter before use.

**Pulse liquid medium**

4.3 g MS salts (e.g., Caisson, cat. no. MSP01)
0.4 ml of 1 mg/ml thiamine HCl
2 ml of 1 mg/ml glycine
100 mg myo-inositol
1 ml of 0.5 mg/ml pyridoxine HCl
1 ml of 0.5 mg/ml nicotinic acid
1.8 g glucose
Adjust pH to 5.7 with KOH
Bring to 1 L with deionized water
Store at 4°C for up to 1 month or at room temperature for up to 2 weeks
On the day of use, supplement 50 ml pulse liquid medium with 125 μl of 100 mM DTT, 50 μl of 1 mg/ml 2,4-D, and 5 μl of 1 mg/ml BAP. Gently mix by inverting the tube five times.
Thiamine HCl is light sensitive; it should be wrapped in foil and stored at 4°C for up to 6 months. Glycine, pyridoxine HCl, and nicotinic acid solutions should be stored as 1-ml aliquots at −20°C for up to 6 months.
DTT, 2,4-D, and BAP solutions should be filter sterilized using a 0.22-μm filter before use.
**Pulse preculture medium**

- 4.3 g MS salts (e.g., Caisson, cat. no. MSP01)
- 0.4 ml of 1 mg/ml thiamine HCl
- 2 ml of 1 mg/ml glycine
- 100 mg myo-inositol
- 1 ml of 0.5 mg/ml pyridoxine HCl
- 1 ml of 0.5 mg/ml nicotinic acid
- 1.802 g glucose
- Adjust pH to 5.7 with KOH
- 7 g agar (e.g., Sigma-Aldrich, cat. no. A1208)

Bring to 1 L with deionized water

Sterilize by autoclaving and cool to 55°C to 60°C in a water bath

Supplement with 2.5 ml of 100 mM DTT, 1 ml of 1 mg/ml 2,4-D, and 0.1 ml of 1 mg/ml BAP

Store at 4°C for up to 1 month or at room temperature for up to 2 weeks

*Thiamine HCl is light sensitive; it should be wrapped in foil and stored at 4°C for up to 6 months. Glycine, pyridoxine HCl, and nicotinic acid solutions should be stored as 1-ml aliquots at −20°C for up to 6 months.*

*DTT, 2,4-D, and BAP solutions should be filter sterilized using a 0.22-μm filter before use.*

**Pulse selective medium**

- 4.3 g MS salts (e.g., Caisson, cat. no. MSP01)
- 0.4 ml of 1 mg/ml thiamine HCl
- 2 ml of 1 mg/ml glycine
- 100 mg myo-inositol
- 1 ml of 0.5 mg/ml pyridoxine HCl
- 1 ml of 0.5 mg/ml nicotinic acid
- 30 g sucrose
- Adjust pH to 5.7 with KOH
- 7 g agar (e.g., Sigma-Aldrich, cat. no. A1208)

Bring to 1 L with deionized water

Sterilize by autoclaving and cool to 55°C to 60°C in a water bath

Supplement with 2.5 ml of 100 mM DTT, 1 ml of 1 mg/ml 2,4-D, 0.1 ml of 1 mg/ml BAP, 0.75 ml of 100 mg/ml kanamycin, and 3 ml of 100 mg/ml timentin

Store at 4°C for up to 1 month or at room temperature for up to 2 weeks

*Thiamine HCl is light sensitive; it should be wrapped in foil and stored at 4°C for up to 6 months. Glycine, pyridoxine HCl, and nicotinic acid solutions should be stored as 1-ml aliquots at −20°C for up to 6 months.*

*DTT, 2,4-D, BAP, kanamycin, and timentin solutions should be filter sterilized using a 0.22-μm filter before use.*

**Rifampicin stock, 12 mg/ml**

- 60 mg rifampicin (e.g., PhytoTechnology, cat. no. R501)

Bring to 5 ml with methanol

Filter sterilize using a 0.22-μm filter

Store 1-ml aliquots at −20°C for up to 3 months

**TAJ liquid medium**

- 4.3 g MS salts (e.g., Caisson, cat. no. MSP01)
- 1 ml 3R vitamins (see recipe)
- 100 mg myo-inositol
- 1.802 g glucose
Adjust pH to 5.9 with KOH
Bring to 1 L with deionized water
Sterilize by autoclaving and cool to 55°C to 60°C in a water bath
Store at 4°C for up to 1 month or at room temperature for up to 2 weeks

On the day of use, supplement 50 ml TAJ liquid medium with 125 μl of 100 mM DTT, 100 μl of 1 mg/ml trans-zeatin (see recipe), and 12.5 μl of 1 mg/ml NAA (see recipe). Gently mix by inverting the tube five times.

DTT, trans-zeatin, and NAA solutions should be filter sterilized using a 0.22-μm filter before use.

**TAJ preculture medium**

- 4.3 g MS salts (e.g., Caisson, cat. no. MSP01)
- 1 ml 3R vitamins (see recipe)
- 100 mg myo-inositol
- 1.802 g glucose
- Adjust pH to 5.9 with KOH
- 8 g agar (e.g., Sigma-Aldrich, cat. no. A1208)

Bring to 1 L with deionized water
Sterilize by autoclaving and cool to 55°C to 60°C in a water bath
Supplement with 0.25 ml of 1 mg/ml NAA (see recipe), 6 ml of 1 mg/ml trans-zeatin riboside (see recipe), and 2.5 ml of 100 mM DTT
Store at 4°C for up to 1 month or at room temperature for up to 2 weeks

NAA, trans-zeatin ribose, and DTT solutions should be filter sterilized using a 0.22-μm filter before use.

**Trans-zeatin, 1 mg/ml**

- 25 mg trans-zeatin (e.g., Caisson, cat. no. Z007)
- Dissolve with a few drops of 0.5 M HCl
- Bring to 25 ml with deionized water
- Filter sterilize using a 0.22-μm filter
- Store 1-ml aliquots at −20°C for up to 6 months

**Trans-zeatin riboside, 1 mg/ml**

- 25 mg trans-zeatin riboside (e.g., Caisson, cat. no. Z008)
- Dissolve with a few drops of 0.5 M HCl
- Bring to 25 ml with deionized water
- Filter sterilize using a 0.22-μm filter
- Store 1-ml aliquots at −20°C for up to 6 months

**YENB liquid medium**

- 7.5 g yeast extract (e.g., BD, cat. no. 212750)
- 8 g nutrient broth (e.g., BD, cat. no. 234000)

Bring to 1 L with deionized water
Adjust pH to 7.5 with KOH
Sterilize by autoclaving
Store at 4°C for up to 1 month or at room temperature for up to 2 weeks

**YEP liquid medium**

- 10 g yeast extract
- 10 g peptone
- 5 g NaCl

Bring to 1 L with deionized water
Adjust pH to 7.0 with KOH
Sterilize by autoclaving
Store at 4°C for up to 1 month or at room temperature for up to 2 weeks

COMMENTARY

Background Information
Among the three species of milkweed studied here, their biology and ecology vary substantially, although all species are herbaceous perennials of open habitats that are largely self-incompatible, and all are hosts for the monarch butterfly (*Danaus plexippus*; Agrawal, 2017). The common milkweed, *A. syriaca* is a highly clonal species abundant in fields, roadsides, and disturbed habitats throughout much of eastern North America. Butterflyweed, *A. tuberosa*, is of shorter stature, not clonal, and is typical of dry or gravelly soil, patchily distributed through the United States except in the Pacific Northwest. Finally, Hall’s milkweed, *A. hallii*, has a restricted range in the western United States (but east of California) at mid elevation (around 2000 meters above sea level). Milkweeds currently have minimal uses in agriculture, although native Americans used them for fiber, food, and medicine. Because milkweeds have long been a model species to understand chemically mediated ecological interactions, and were the first system in which sequestration of plant toxins by an insect herbivore was demonstrated, a transformation system would be highly beneficial to advancing basic research of these abundant and conspicuous species.

Availability of gene transfer methods for these three different *Asclepias* species provides a tool that can be combined with other approaches to dissect the genetic control over their metabolites and insect interactions. One approach for gene function studies that relies on established gene transfer methods is genome editing, which is a valuable technology for precise modification of genes of interest (Van Eck, 2018). Transformation methods are needed for introduction of gene editing components into plant cells. Therefore, with the methods described in this article, gene editing of these *Asclepias* is now possible. As a note, being that the three species used for development of the method are self-incompatible, the seeds used to generate plant material for tissue culture originated from open-pollinated plants. As a result, there is variation among the progeny in each species.

In the early developmental stages of this method, we encountered a significant incidence of explants turning brown (oxidation), which led to loss of entire experiments. After testing various medium supplements that included activated charcoal and antioxidants such as ascorbic acid, we found that inclusion of freshly prepared DTT was most effective at preventing oxidation. Once we were able to prevent browning of the explants, we recovered transgenic lines for each species at the following transformation efficiencies: *A. hallii*, 9%; *A. syriaca*, 6%; and *A. tuberosa*, 13%.

Another challenge has been finding the best conditions for rooting transgenic lines. Our primary focus has been with rooting of *A. syriaca* transgenic lines. We presented two methods for rooting: rooting in soil and use of the EZ Clone system. While the EZ Clone approach is still in the early stages, we have recovered a higher percentage of rooted shoots (42%) in a shorter period of time (3 weeks) compared with other approaches we have tested. If the EZ Clone system is not available, rooting in soil is possible, and the following factors play an important role in rooting success: bottom heat, rooting hormones when transferred to soil, shading, maintenance of high humidity, misting of rooting area, and seasonality, with the best results occurring in the fall in upstate New York. Although we have focused our efforts on *A. syriaca*, these methods can be applied to rooting of *A. hallii* and *A. tuberosa*.

Critical Parameters
DTT needs to be freshly prepared at the time of each use. We have found that it is less effective at preventing oxidation of explants if stored for any length of time.

The maximum time media should be stored at room temperature is 2 weeks. For best practices, media should be prepared as close as possible to time of use.

Latex that often exudes from the explants appears as a faint white halo in the medium.

Troubleshooting
In vitro rooting of *A. syriaca* and *A. hallii* has not been achieved despite investigation of various parameters. *A. tuberosa* has occasionally produced tuberous roots in vitro; however, these results were sporadic. Therefore, rooting in soil or hydroponically by the Clonex method as outlined in Basic Protocol 1 is recommended for all species.
Explants not inoculated with *Agrobacterium* serve as controls. The explants are evenly divided between selective medium and medium that does not contain selection agents to serve as negative and positive controls, respectively.

Stem cuttings turn brown when cultured on medium. Addition of freshly prepared DTT to the medium greatly reduces this.

Depending on the *Asclepias* species, the first groups of transgenic lines are at the best developmental stage for rooting in soil between 3 and 5 months after inoculation of explants with *Agrobacterium*. Following verification by PCR that lines are indeed transgenic, cuttings of positive lines can be taken and maintained on ESM for *A. hallii* and *A. tuberosa* and on TAJ medium for *A. syriaca* before transfer to soil for rooting. Rooting may be a lengthy process taking several months from time of transfer to soil.

**Time Considerations**

The following is a recommended general timeline for transformation of each species:

For *A. hallii* and *A. tuberosa*, sterilize and scarify seeds 3.5 to 4 weeks prior to the anticipated transformation date, allowing for 2 to 3 weeks of growth. To establish in vitro plants of *A. syriaca* for transformation, prepare seeds for germination 9 weeks before scheduled transformation experiments. This timeframe includes 2 weeks for growth and 6 weeks to establish in vitro plants.

For *A. syriaca*, the coculture period is 7 days, whereas the coculture period for *A. hallii* and *A. tuberosa* is 2 days. Maintain in the dark at 22°C over the weekend. Transfer all cultures to freshly prepared medium every 2 weeks.

After ~2 to 4 months, putative *A. hallii* and *A. tuberosa* transgenic lines can be transferred to ESM and *A. syriaca* to plant development selective medium. PCR analysis should be performed to verify presence of the transgene.

Take cuttings of verified lines to propagate in vitro. At ~3 to 5 months after initiation of transformation, transgenic lines can be transferred to soil. Periodically assess for rooting.

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**Literature Cited**


