Protocol

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**3. Dates of visit**: June 13, 2011 – Aug 19, 2011
**4. Title**: Genetic Transformation of *Cornus canadensis* using *Agrobacterium* suspension
**5. Rationale and Background**: Described is a method of producing transgenic *Cornus canadensis* plants by *Agrobacterium* infiltration. Aside from basic laboratory equipment and other basic materials described below, this protocol presupposes the possession of two materials beforehand: a) a viable *Agrobacterium* line with vector containing the transgene in question; b) a vigorous, sterile tissue culture line with enough growing plantlets to harvest explants.
**6. Protocol**:

# Overview

1. Activate *Agrobacterium*

The *Agrobacterium* carrying the desired vector is removed from freezer storage and allowed to resume growth on a nutrient-filled petri dish. All transformation experiments using this particular *Agrobacterium* and vector will begin the culture of their *Agrobacterium* suspension from this dish.

2. Prepare Plates

Petri dishes (herein usually referred to as 'plates') are the containers and basic unit of each experiment. Plates are filled with a plant hormones, nutrients, and antibiotics dissolved in an agar gel. This is the growing medium for the plants. Depending on the hormones used, plants can be induced to regenerate, form a callus, grow shoots, or grow roots. Because vectors often include a transgene for antibiotic resistance, including high concentrations of antibiotics in the plates will kill non-transformed plants while leaving transformed plants unaffected.

3. Explant Pre-culture

Tiny sections of leaf are harvested from sterile cultures of regenerated plants and placed on regeneration plates. These fragments of leaf tissue are the explants, and each has the potential to regenerate into a complete plant. Each explant is an individual experimental subject upon which genetic transformation will be attempted. Generally a great many individual explants are used because the chance of a successful transformation is very small. This protocol is written based on the use of eight plates per experiment with 25 explants per plate.

4. Prepare *Agrobacterium*

To effectively infiltrate plant cells and transfer the transgene they carry, a liquid *Agrobacterium* suspension of specific parameters must be prepared. Concentration of individual microbes, population growth rate, nutrient density of medium, antibiotic concentration of medium, and other factors must be considered and controlled for to effect a transformation.

5. Co-culture

Explants are immersed in the *Agrobacterium* suspension for a short time. They are then washed with clean water and allowed to sit in darkness for several days while the *Agrobacteria* infiltrate the cells.

6. Resting

Explants are washed and placed on a callus induction medium, which causes individual cells to multiply into undifferentiated plant tissue called a callus. This medium also includes antibiotics to which *Agrobacterium* is not resistant, thus killing them. The explants rest in the dark for a week to recover from co-culture shock.

7. Selection

Antibiotics in the selection medium allow only transformed cells to survive and grow. Over several weeks these transformed cells divide and grow stronger while non-transformed cells die out, eventually leaving only plants carrying the transgene of interest.

# Protocol

## 1. Activate *Agrobacterium*

1. Remove *Agrobacterium* stock strain from -80° C freezer.
2. Using a pipette tip, transfer a small sample of bacterium to a YEP plate. Replace stock strain in freezer.
3. Incubate YEP plate at 28° C for 2 days. After this, store in 4° C refrigerator All further experiments using this particular *Agrobacterium* and vector uses samples from this plate. Activation from freezer storage is necessary only if this plate culture does not exist.

## 2. Prepare MS Plates

1. **Prepare MS medium**
    1. Gather:
        - large mixing container
        - Erlenmeyer flasks for each different medium
        - 1000uL pipette and tips
        - 20mL graduated cylinder
        - MS chemicals (from refrigerator, 4° C)
    2. Wash mixing container, flasks, and graduated cylinder, then rinse with milliQ water. Fill mixing container with milliQ H2O to nearly the total volume (ie add 1900mL when preparing 2L MS medium).
    3. Pipette chemicals requiring 1-2mL each from each stock solution into mixing container. After each transfer, discard pipette tip and tightly close stock container.
    4. For chemicals requiring 10-20mL each, pour carefully from stock container into graduated cylinder, then into mixing container. Then tightly close stock container.
    5. Add sucrose and phyto agar to Erlenmeyer flasks
    6. Add a clean magnetic stirbar to mixing container and begin stirring. Rinse pH probe with dH2O and place into mixing container. Adjust pH to 5.8 using a pipette and dropwise addition of 1M NaOH (and 1M HCl if needed).
    7. Pour from the mixing container to equally fill each of the Erlenmeyer flasks. Do not fill flasks more than halfway. If using a flask with a cap, ensure cap is very loose. Cover flask mouth with aluminum foil and secure with autoclave-tape. Place flasks in a metal tray.
2. **Autoclave** - Autoclave flasks for 30 minutes on liquid cycle. Remove flasks from autoclave as soon as practical. Do not let flasks cool in autoclave.
3. **Gather**:
    - autoclaved flasks filled with MS medium
    - sterile petri dishes (1 plate / ~30 mL)
    - any hormones or antibiotics necessary
    - pipette
    - sterile pipette tips
    - 70% EtOH spray bottle
    - 70% EtOH refill bottle
    - gloves
    - sleeves

    - marker
    - paper towels
4. **Sterilization and Preparation** - Don gloves and sleeves, and spray alcohol on them. Spray work space and wipe down with paper towels. Transfer materials (except for spray bottle, refill bottle, and paper towels) to flow hood, spraying them beforehand. Open the plastic cover of the sleeves of petri dishes and place on hood work surface, bottom down, with cover atop.
5. **Hormone and Antibiotic Addition -** Swirl flasks well. When temperature is below ~50° C (varies depending on hormone and antibiotic used), remove foil from flasks, swirl again, and add appropriate hormones and antibiotics necessary for each medium. Re-cover flasks with aluminum foil used during autoclaving.
6. **Pour the Plates** - Remove stack of up to 10 dishes from plastic sleeve. Remove foil from appropriate flask and swirl well once again. Starting with the bottom-most plate, lift the lid off with the other plates balanced on top. With your other hand, swiftly pour the medium into the plate until the bottom is covered, and then replace the lid. Set the plate stack down on top, lift the next lid up with remaining plates above it, and repeat, working your way up the stack until each plate in the stack is filled. Repeat for each flask of medium. For media upon which explants will be resting for only a short time, one can pour medium into a plate until it is perhaps 75% covered, and later tip the plate so that the medium covers the entire plate. In this way many more plates can be filled. It is best to time this operation such that the solution is as cool as possible when being poured without having already begun to solidify. This will keep excess moisture from condensing on the plate lids.
7. **Cleanup -** Be sure to keep plates of different media separate while pouring. When finished, re-cover the stack with the plastic sleeve the plates came in, and label clearly. If the hood work surface is not level, use a pipette tip to prop up the stack of plates so that when the agar sets, it is as level as possible. After they have fully solidified, roll up excess plastic covering and secure with tape. Place any plate stacks which will not be used in the next day or two in the refrigerator

# 3. Explant Pre-culture

1. **Gather**:
    - regeneration medium plates
    - plates with young, vigorous plantlets growing (footnote)
    - several empty, unused plates
    - parafilm strips, one for each plate
    - scalpel, autoclaved
    - forceps, autoclaved
    - 100mL beaker
    - 70% EtOH spray bottle
    - 70% EtOH refill bottle
    - lighter
    - alcohol lamp
    - gloves
    - sleeves
    - marker
    - paper towels
2. **Sterilization and Preparation** - Don gloves and sleeves, and spray alcohol on them. Spray work space and wipe down with paper towels. Fill beaker 1/3 to 1/2 full with

70% EtOH. Transfer materials (except for spray bottle, refill bottle, and paper towels) to flow hood, spraying them beforehand. Light lamp, thoroughly flame forceps and scalpel. Remove a number of regeneration plates from their plastic sheet sufficient to hold the number of explants you wish to transform. Each plate can hold many explants, but the standard number per plate is 25. Reseal the plastic sheet after removing those you will use. Remove parafilm from plates holding growing source plantlets.

3. **Harvest Explants -** Place one half of one of the empty, unused plates in front of you to be used as a cutting area. Remove the lid from one of the regeneration plates. Flame the forceps and scalpel, and transfer one of the plantlets to the cutting plate. Select a young leaf and sever it from its shoot. If it is large enough, slice it in half laterally. You are aiming for explants approximately 0.2 cm x 0.2 cm in size. Gently transfer each explant with forceps or end of scalpel to the regeneration medium, pressing it in slightly. Repeat the process until the regeneration plate has 25 explants, then stow the scalpel and forceps in the EtOH well, set aside the filled regeneration plate, and open the next one. You will frequently have to rinse the scalpel and forceps in the EtOH well to rid them of tiny bits of plant matter; always flame off the alcohol afterward. Switch out cutting plates when they become messy, and obtain new source plantlets when there is no suitable material left on old ones.
4. **Cleanup -** Wrap the edges of all filled regeneration plates with parafilm and label each one. If there is usable material left in the source dishes, parafilm these as well. Discard all used source material and cutting dishes. Remove EtOH well, forceps, scalpel, regeneration plates, and source plates. Spray down work area with EtOH and wipe with paper towel. Turn off hood lamp; keep fan on. Replace any unused regeneration plates in the refrigerator
5. **Pre-Culture -** Place regeneration plates with explants under a weak lamp at 23° C and let grow for three days.

## 4. Prepare *Agrobacterium*

1. ***Agrobacterium* 2uL culture -**
    1. Gather:
        - an active plate from the 4° C fridge
        - pipette
        - sterile pipette tips
        - ~2mL microcentrifuge tube
        - 10-20 mL test tube
        - liquid YEP medium
        - parafilm strip
    2. Under a flow hood, pipette 1mL YEP medium to 2mL microcentrifuge tube. Remove parafilm from *Agrobacterium* plate, and scrape off a small amount of bacterium using a fresh pipette tip. Transfer this to the YEP medium in the 2mL microcentrifuge tube, and mix well. Pour into the 10mL test tube, and then pipette in an additional 1mL YEP medium. Cap 10mL tube in hood, label, and incubate at a slant with shaking at 28° C overnight.
2. ***Agrobacterium* 50uL culture -**
    1. Gather:
        - 10mL test tube containing 2mL *Agrobacterium* culture from incubator
        - 50mL liquid YEP medium
        - autoclaved 250mL Erlenmeyer flask
    2. Under a flow hood, agitate 2mL *Agrobacterium* culture well and transfer to 250mL Erlenmeyer flask. Then transfer 50mL liquid YEP medium, rinsing the 10mL test

tube with medium if desired. Recover the 250mL Erlenmeyer flask with the aluminum foil it was autoclaved with. Label and place into incubator at 28° C overnight with stirring.

3. **Prepare *Agrobacterium* pellets -**
    1. Check the culture OD600 value at a spectrophotometer
        1. Gather:
            - 2 cuvettes
            - liquid YEP medium identical to that used for bacterial culture (same antibiotics)
            - pipette
            - sterile pipette tips
            - 50 mL *Agrobacterium* culture
        2. Under a flow hood, pipette 600-1000 mL liquid YEP medium to one cuvette. This will be the control used to zero the spectrophotometer. Agitate the *Agrobacterium* culture well, and pipette 600-1000 mL into the second cuvette.
        3. Turn on spectrophotometer. Place cuvettes in with arrow side towards your right, with the control at the well labeled 1 and the *Agrobacterium* sample at well 2. Close spectrophotometer cover. Adjust wavelength to 600 nm. Zero the spectrophotometer on the control, then move it to the sample. We are aiming for an OD value of 0.8, though between 0.8-1.0 is acceptable.
    2. If OD value is higher than 1.0, add more liquid YEP to the *Agrobacterium* culture, return culture to shaking incubator, and check the new OD value after an hour. If OD value is lower than 0.8, return *Agrobacterium* culture to incubator and check again in an hour. If OD value is within desired range, gather two 50mL screw-top centrifuge tubes, and, in the flow hood, partition the 50mL *Agrobacterium* culture between the two centrifuge tubes, capping them securely afterward.
    3. Centrifuge - Turn on centrifuge cooler to 4° C. This can be done before measuring the culture OD value. After centrifuge has reached 4° C, place centrifuge tubes with *Agrobacterium* culture in cradles on opposite sides of centrifuge, balancing them. Centrifuge at 4000 rpm for 10 minutes.
    4. Remove tubes from centrifuge, pour off supernatant liquid into a waste beaker in flow hood, and pour in a small amount of bleach to destroy the genetically modified bacterium. Recap tubes with bacterium pellet at bottom to be used in next step.

# 5. Co-culture

1. **Gather**:
    - co-culture medium plates
    - liquid MS medium
    - centrifuge tubes with *Agrobacterium* pellets at bottom
    - autoclaved H2O
    - autoclaved filter disks
    - an empty 50mL centrifuge tube
    - several empty, unused plates
    - parafilm strips, one for each plate
    - forceps, autoclaved
    - 100mL beaker
    - 70% EtOH spray bottle
    - 70% EtOH refill bottle
    - lighter

- alcohol lamp
- gloves
- sleeves
- marker
- paper towels

2. **Sterilization and Preparation** - Don gloves and sleeves, and spray alcohol on them. Spray work space and wipe down with paper towels. Fill beaker 1/3 to 1/2 full with 70% EtOH. Transfer materials (except for spray bottle, refill bottle, and paper towels) to flow hood, spraying them beforehand. Light lamp, thoroughly flame forceps. Remove a number of co-culture plates from their plastic sheet equal to the number of pre-culture regeneration plates you are going to transform. Reseal the plastic sheet after removing those you will use.
3. **Prepare *Agrobacterium* Suspension** - Pour liquid MS medium onto *Agrobacterium* pellets in centrifuge tubes up to level of previous YEP volume (usually ~25-30mL). Close and shake vigorously until no trace of *Agrobacterium* cake remains at bottom. Pour an equal amount of liquid MS medium into another tube for use as a control.
4. **Immerse Explants** - Remove parafilm from three regeneration plates for the first batch of *Agrobacterium* innoculant. Retrieve a clean, sterile, unused plate. Shake an *Agrobacterium* vial and pour into the bottom half of the clean plate. Use forceps to transfer explants from regeneration plates into *Agrobacterium* innoculant, pushing them so that all leaf surfaces come in contact with innoculant. When complete, cover *Agrobacterium* suspension with the top half of the clean plate and agitate explants 2-3 times over the next 15 minutes. Set aside empty pre-culture plates to be disposed of.
5. **Wash Explants -** When 15 minutes have nearly passed, remove the lid of the plate holding the *Agrobacterium* suspension and fill it nearly to the top with autoclaved H2O. Transfer the explants swimming in *Agrobacterium* to the clean, sterile water; agitate them so that all their surfaces are rinsed. When all are transferred, retrieve a number of co-cultivation plates equal to the number of regeneration plates used to fill the bacterium innoculant dish. Take also a piece of autoclaved filter disk for drying. With the forceps, remove the explants from the rinse water, touch them to the filter disk to dry excess water, and then place them on the co-cultivation plates, pressing them slightly into the medium.
6. **Repeat** - Repeat steps 4 and 5 for other *Agrobacterium* tube and the control. Generally three plants of explants are immersed in the suspension from one of two *Agrobacterium* tubes, while two plates of explants are immersed in the liquid MS control.
7. **Cleanup and Co-culture** - Parafilm and label the co-cultivation plates. Clean work area with alcohol. Dispose of the suspension and any materials that touched the *Agrobacterium* in a hazardous waste bin (to be autoclaved). Let co-cultivation plates incubate in the dark at 23° C for 3 days.

# 6. Resting

1. Gather:
   - resting medium plates
   - autoclaved H2O
   - autoclaved filter disks
   - several empty, unused plates
   - parafilm strips, one for each plate
   - forceps, autoclaved
   - 100mL beaker
   - 70% EtOH spray bottle

    - 70% EtOH refill bottle
    - lighter
    - alcohol lamp
    - gloves
    - sleeves
    - marker
    - paper towels
2. **Sterilization and Preparation** - Don gloves and sleeves, and spray alcohol on them. Spray work space and wipe down with paper towels. Fill beaker 1/3 to 1/2 full with 70% EtOH. Transfer materials (except for spray bottle, refill bottle, and paper towels) to flow hood, spraying them beforehand. Light lamp, thoroughly flame forceps. Remove a number of resting plates from their plastic sheet equal to the number of co-culture plates there are. Reseal the plastic sheet after removing those you will use. Remove parafilm from co-culture plates.
3. **Wash** - Place one side of an empty, unused plate on the work surface and fill with autoclaved H2O. Flame the forceps, remove the lid of one co-culture plate, and transfer all explants into the water. Agitate the explants to ensure all sides are exposed to the wash water. Remove the lid of a resting plate. Remove the explants from the water wash, dry them on a filter disk, and then press them into the resting medium plate. When complete, cover the resting medium dish, push the spent co-culture plate aside, and repeat this process for each co-culture plate you have.
4. **Cleanup and Rest** – Clean work area with alcohol. Remove all wash water to a hazardous waste bin to be autoclaved. Incubate resting plates in the dark at 23° C for seven days.

# 7. Selection

1. Gather:
    - selection medium plates
    - autoclaved H2O
    - autoclaved filter disks
    - several empty, unused plates
    - parafilm strips, one for each plate
    - forceps, autoclaved
    - 100mL beaker
    - 70% EtOH spray bottle
    - 70% EtOH refill bottle
    - lighter
    - alcohol lamp
    - gloves
    - sleeves
    - marker
    - paper towels
2. **Sterilization and Preparation** - Don gloves and sleeves, and spray alcohol on them. Spray work space and wipe down with paper towels. Fill beaker 1/3 to 1/2 full with 70% EtOH. Transfer materials (except for spray bottle, refill bottle, and paper towels) to flow hood, spraying them beforehand. Light lamp, thoroughly flame forceps. Remove a number of selection plates from their plastic sheet equal to the number of resting plates there are. Reseal the plastic sheet after removing those you will use. Remove parafilm from resting plates.

3. **Wash -** Place one side of an empty, unused plate on the work surface and fill with autoclaved H2O. Flame the forceps, remove the lid of one resting plate, and transfer all explants into the water. Agitate the explants to ensure all sides are exposed to the wash water. Remove the lid of a selection plate. Remove the explants from the water wash, dry them on a filter disk, and then press them into the selection medium plate. When complete, cover the selection medium dish, push the spent resting plate aside, and repeat this process for each resting plate you have.
4. **Cleanup and Selective Regeneration** – Clean work area. Incubate selection plates under mild grow lights with a photoperiod of 14/10 (light hours/dark hours) at 23° C for three weeks.
5. **Repeat** – Repeat this selection step, transferring surviving calli to fresh selection dishes every three weeks until all control calli are dead.
6. **Elongate, Root, and Transplant** – After the only surviving calli are transformed, they can be elongated, rooted, and then transplanted to non-sterile area following standard tissue culture protocols as used to obtain original tissue line.

## 7. Recipes and Stock Solutions:

**Liquid YEP medium**

10 g yeast extract
10 g Bacto Peptone
5 g NaCl

Adjust pH to 7.0, and bring final volume to 1 liter with H2O. Autoclave and store in 4° C refrigerator Before use, add antibiotics at the following concentrations:

25 mg/l rifamycin
50 mg/l kanamycin

**YEP plate**

10 g yeast extract
10 g Bacto Peptone
5 g NaCl
8 g phyto-agar
25 mg rifamycin
50 mg kanamycin

Adjust pH to 7.0, and bring final volume to 1 liter with H2O. Autoclave, let cool to 30-40° C, and add antibiotics and pour plates as described in the "Prepare MS Plates" section.

**Regeneration Medium**

1000 mL MS medium
30 g sucrose
8 g phyto-agar

As described in the "Prepare MS Plates" section, adjust pH to 5.8, autoclave, let cool to 30-40° C, and then add hormones:

100 uL NAA
1000 uL BAP

**Co-cultivation Medium**

1000 mL MS medium

30 g sucrose
8 g phyto-agar

As described in the "Prepare MS Plates" section, adjust pH to 5.8, autoclave, let cool to 30-40° C, and then add hormones:

100 uL NAA
1000 uL BAP
40 uL acetosyringone

**Liquid MS medium**

1000 mL MS medium
30 g sucrose

As described in the "Prepare MS Plates" section, adjust pH to 5.8, autoclave, and refrigerate at 4° C. When ready for use, add hormones:

40 uL acetosyringone

This solution with acetosyringone is not stable and should not be stored for long periods. Therefore, in practice, the liquid MS medium is stored without acetosyringone, and acetosyringone is added to a small volume of MS medium as needed for creating the *Agrobacterium* suspension:

20 mg acetosyringone dissolved in 40 uL DMSO for 1000 mL Liquid MS medium
1mg acetosyringone dissolved in 2 uL DMSO, for 50 mL Liquid MS medium

**Resting Medium**

1000 mL MS medium
30 g sucrose
8 g phyto-agar

As described in the "Prepare MS Plates" section, adjust pH to 5.8, autoclave, let cool to 30-40° C, and then add hormones and antibiotics:

100 uL NAA
1000 uL BAP
40 uL acetosyringone
630 uL Timentin

**Selection Medium**

1000 mL MS medium
30 g sucrose
8 g phyto-agar

As described in the "Prepare MS Plates" section, adjust pH to 5.8, autoclave, let cool to 30-40° C, and then add hormones and antibiotics:

100 uL NAA
1000 uL BAP
40 uL acetosyringone
2000 uL Kanamycin
630 uL Timentin

**Acetosyringone:** 20 mg acetosyringone dissolved into 40 uL DMSO. Added to 1L solution, this results in a 100uM concentration. This solution should be prepared as needed and should not be stored for significant time.

**Timentin** (3.1g/13mL = 238.46 mg/mL)
**Rifamycin** (25 mg/mL)
**Kanamycin** (100 mg/mL)

**NAA** (1 mg/mL)
**BAP** (1 mg/mL)