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Chapter 6.3

TRANSFORMATION-REGENERATION PROCEDURE FOR *LOTUS JAPONICUS*

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Lotus japonicus *has gained considerable attention because of its increasing use as a model legume. It is thus important to have an effective transformation and regeneration procedure to allow the transfer of foreign genes into Lotus for complementation experiments and the studies of regulation of nodulation and developmental processes.* Agrobacterium tumefaciens *mediated transformation is preferred because of its simplicity and efficiency in providing stable integration of transferred DNA into the plant genome.* In vitro *shoot regeneration of* Lotus japonicus *has been reported from hypocotyl explants (Thykjaer* et al., *1998). The objective of this work was to develop an improved* in vitro *regeneration and an* Agrobacterium tumefaciens *transformation method for* Lotus japonicus *hypocotyl explants.*

EXPERIMENTAL PROCEDURES

Plant material and transformation

Germination

1. Transfer the seeds to be germinated in a container resistant to sulfuric acid.
2. Cover the seeds with sulfuric acid (95-97%) and let them scarify for 2 to 3 min.
3. Wash the seeds 4 times with distilled water
4. Cover the seeds with commercial bleach (8-12% active chloride) and let them set for 20 minutes. The commercial bleach is diluted 20 times (1ml in 20 ml of distilled water plus Tween20 (5 µl))
5. Wash the seeds 5 times with sterile water.
6. In a sterile Petri dish, put a few layers of sterile filter paper.

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7. Wet the filter paper with sterile water and leave a film of water at the bottom.
8. Put the seeds on the top of the filter paper and spread them.
9. Let the seeds germinate at 26°C in continuous light for 3 days or until roots are about 0.5 cm long.

Geminate seeds for 1 week in continuous light at 26 °C (see surface sterilisation protocol). Streak the *Agrobacterium* strains on fresh selective plates. Start 2 to 3 days before transformation (it depends how fast the *Agrobacterium* strain grows). Prepare 2 to 3 Petri dishes for each strain to get enough cells to resuspend in YMB medium. Scrape 2-day-old *Agrobacterium* colonies off selective plates with a sterile toothpick or sterile spatula, and resuspend each strain to a density of approximately 5×10^9 per millilitre in 5 ml of YMB medium by whirling. Leave bacteria as milky cultures at room temperature while preparing the co-cultivation plates.

Prepare co-cultivation plates by placing 0.5 cm-high platforms of sterile filter papers in Petri dishes. Add co-cultivation medium until free liquid appears (12-15 ml). Prepare a new set of Petri dishes with two layers of sterile filter paper. Soak filter papers with 4 ml of *Agrobacterium* YMB suspension or 4 ml of YMB for control plates. Free liquid should appear on the surface of filter paper.

Transfer some seedlings to the *Agrobacterium* filters and using scalpel and forceps cut the seedlings below the shoot primordia and at the stem base. Once cut transversely, hypocotyls are cut longitudinally into two pieces to have more surface contact with *Agrobacterium* and more room for calli to form. Leave the cut hypocotyls soaking in *Agrobacterium* solution into 5 ml containers for approximately 30 min. Transfer the 3 mm hypocotyls pieces into the co-cultivation plates. Seal the Petri dishes with Parafilm but remember to perform some opening within the Parafilm with a sterile scalpel.

Incubate co-cultivation plates for 7 days at 21°C in the dark.

Regeneration procedure

Plant cells are totipotent, i.e., a single cell can give rise to an entire organism provided it is supplied with appropriate nutrients and stimulated with proper combinations of hormones to grow and differentiate. Thus, it is important to ensure the right media and concentration of growth factors.

To eliminate *Agrobacterium*, transfer explants with the top filter paper from the co-cultivation plates to regeneration medium with 300µg/m of cefotaxime. Leave for 5 days to allow the regeneration of the explants.

Start the selection of transgenic calli by including one of the following antibiotics: G418 (25 µg/ml), hygromycin (15 µg/ml). Transfer the explants with the filter paper to selective medium containing 300 µg/ml of cefotaxime. Incubate for 7 days at 26°C in continuous light. (If you have the kanamycin resistance gene in your construct, use Geniticin (G418) instead of kanamycin to select transformed calli, because it is more effective in Lotus. If you can't get calli with 25 µg/ml Geniticin, lower the concentration to 15 µg/ml. In either case, use a kill curve to determine which concentration is effective.)

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Continue selection of transgenic calli by transferring explants, without filter paper to selective medium containing 300 µg/ml of cefotaxime and the appropriate antibiotic.

Continue selection and propagation of transgenic calli on callus medium for 5 weeks. Transgenic green calli are separated from explants as soon as possible and propagated in one piece. Keep transferring the calli to a new media each week.

Start shoot induction by transferring green transgenic calli to shoot induction medium with 300 µg/ml cefotaxime. Shoot structures will develop in the dark green zone appearing at the perimeter of the calli. Keep calli undivided; remove brown and light yellow tissue.

Continue propagation of transgenic calli on shoot induction medium for 3 weeks. Maintain counter selection of *Agrobacterium* with 300 µg/ml cefotaxime until the transfer to the shoot elongation medium. Keep transferring the calli to new shoot induction media each week.

Transfer calli with emerging shoot structures to shoot growth medium for 2 weeks. Keep transferring emerging shoots to new growth media each week.

Transfer calli with emerging shoot structures to shoot elongation medium for 2 weeks. Keep transferring emerging shoots to new elongation media each week.

Cut off individual shoots 2-4 cm long and move to root induction medium for 5 days or until you see the swelling of the base of the shoot (5 to 12 days) at 21°C with a photoperiod of 8/16 hours. Alternatively and based on the observation that cut Lotus shoots easily develop roots in a humid atmosphere, we strongly recommend moving directly the cut shoots to the greenhouse. Put them in very wet soil (without any fertilisers), spray them with water and put them in the minicaphouse. Remember to cover to ensure high level of humidity.

Move shoots to root elongation medium and incubate for 2 weeks or longer at 21°C until the root system is developed enough to be transferred to the greenhouse. Move the shoots with roots into pots containing soil, cover with a plastic bag or use the minicaphouse. Remove the plastic bag gradually, so plants adapt to the new surroundings.

Plant media

Co-cultivation medium

- 1/10 x Gamborg's B5 medium without sucrose
- 5 mM MES, pH 5.2
- 1/10 x B5 vitamins
- NAA (0.05 µg/ml)
- BAP (0.5 µg/ml)

Mix 387 mg ready-made B5 in 1 litre of water and autoclave. Before use, add 5 ml of autoclaved 1M MES, pH 5.2 stock solution, 100 µl of the vitamin stock for B5 medium, 100 µl of NAA, and 1 ml of BAP.

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Regeneration medium

- MS with 2% sucrose
- 0.2% GelRite
- 0.05 µg/ml NAA
- 0.5 µg/ml BAP
- 300 µg/ml cefotaxime

For 1 litre, add 3.87 g of ready-made B5, 20 g of sucrose, and 2 g of GelRite. Adjust pH to 5.5 and autoclave. This is called LO medium. Add 1 ml of the vitamins for B5 medium, 1 ml of BAP, and 100 µl of NAA before pouring cooled medium into 9 cm Petri dishes.

Selection medium

Add the appropriate antibiotic to regeneration medium.

Shoot induction medium

- MS with 2% sucrose
- 0.2% GelRite
- 10 mM NH_4^+
- BAP at 0.5 µg/ml

Prepare 1 litre LO medium as above, then add 2.5 ml of autoclaved 2M $(NH_4)_2SO_4$ to hot medium. Then add 1 ml of BAP stock solution and 1 ml of the vitamin stock for B5 medium to cooled medium. Pour into Petri dishes.

Shoot growth medium*

- B5 with 2% sucrose
- 0.2% GelRite
- BAP at 0.2 µg/ml

Prepare 1 litre LO as above, then add 400 µl of BAP stock solution and 1 ml of the vitamin stock for B5 medium to cooled medium. Pour into plant containers.

Shoot elongation medium*

- B5 with 2% sucrose
- 0.2% GelRite
- BAP at 0.05 µg/ml

Prepare 1 litre LO as above, add 1 ml of the vitamin stock solution for B5 medium and 80 µl BAP and pour into plant containers. (If your shooting plants are looking fine, you can simply use half-strength B5 with only vitamins and no BAP.)

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*****_For shoot growth and elongation medium, we replace MS with B5. On B5, shooting improves and calli make proper shoots, not just leaves._

Root induction medium

- Half-strength B5 with 1% sucrose
- 0.4% GelRite
- 0.5 to 1 µg/ml NAA

For 1 litre, add 1.94 of B5, 10 g sucrose, and 4 g of GelRite and autoclave. This is called L medium. Before use, add 500 µl of vitamin stock solution and the right amount of NAA depending in the concentration. Pour into plant containers.

Root elongation medium

- Half-strength B56 medium with 1% sucrose and 0.4% GelRite.

Use 1 litre L medium with 500 µl of the vitamin stock solution for B5 medium and pour into plant containers.

Agrobacterium media

Solid agar plates

- 0.5% yeast extract
- 1% bacto-tryptone
- 0.5% Nacl
- 1.4% agar, pH 7

For 1 litre, add 5 g of yeast extract, 10 g of bacto-tryptone, 5 g of NaCl, and 14 g of agar. Adjust pH to 7 and autoclave. Stored medium is remelted in a microwave oven and cooled. Antibiotics for selecting appropriate binary T-DNA vectors are added before plates are poured.

YMB liquid growth medium

- 0.2% Mannitol
- 0.04% Yeast extract
- 0.02% $MgSO_4 7H_2O$
- 0. 01% NaCl

For 1 litre, dissolve 2 g mannitol, 0.4 g yeast extract, 0.2 g $MgSO_4 7H_2O$ and 0.1 g NaCl and autoclave. Autoclave a 6.55% $K_2HPO_4 3H_2O$, pH 6.8 solution separately, add 10 ml per litre before use. This media is used to resuspend *Agrobacterium* for transformation.

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Seedling media

Solutions for sterilisation and germination of seeds

- 2% hypochlorite
- 0.02% Tween 20

For 20 ml, mix 2.7 ml of a 15% sodium hypochlorite solution with autoclaved water in a sterile universal container. Add 4 µl Tween20, shake gently, and use the same day.

Support material in liquid cultures

Squares of filter paper (6 x 6 cm) are placed in aluminium foil and autoclaved.

Solutions and media for co-cultivation, selection of transgenic calli, and plant regeneration

Plant hormone stock solutions

- BAP at 0.5 mg/ml: Dissolve 25 mg BAP in 1 ml 1M NaOH and add water to 50 ml
- NAA at 0.5 mg/ml: Dissolve 25 mg NAA in 24 ml 96% ethanol and add water to 50 ml. All solutions are filter sterilised into 2 ml aliquots and stored at -20°C. Upon use, you can heat gently in a microwave oven.

Antibiotic stock solutions

- Kanamycin at 100 mg/ml: Dissolve 2.5 g kanamycin in 25 ml H2O, filter sterilise into 5 ml aliquots and store at -20°C.
- G418 (Geniticin) at 50 mg/ml: Dissolve 1.25 g G418 in 25 ml water. Filter sterilise into 5 ml aliquots and store at -20°C.
- Cefotaxime at 30 mg/ml: For 1 litre of medium, dissolve 300 mg cefotaxime (claforan) in 10 ml H_2O, filter-sterilise and use the same day or you can prepare a stock and keep at -20°C in 10 ml containers.

Other solutions

- 1M MES: Dissolve 4.88 g in 25 ml of water, adjust pH to 5.2 with KOH. Autoclave before use.
- 2M$(NH_4)_2SO_4$: Dissolve 198.24 g in 250 ml. Autoclave before use.

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