

## Chapter 6.1

### AGROBACTERIUM-MEDIATED *IN VITRO* TRANSFORMATION

Patrizia Lombari, Emanuela Ercolano, Hicham El Alaoui, and  
Maurizio Chiurazzi\*

*International Institute of Genetics and Biophysics; Via Marconi 12; 80125, Napoli; ITALY; \*Corresponding author.*

Email: [chiurazz@igb.cnr.it](mailto:chiurazz@igb.cnr.it)

Phone: +39 081 725 7256 Fax: +39 081 593 6123

Keywords: organogenesis, *in vitro*, Agrobacterium, transformation, regeneration

*A major obstacle for understanding the molecular basis of root nodule morphogenesis has been for many years the lack of a well-developed genetic system in a legume that is amenable to the advanced technologies developed for model plants such as Arabidopsis thaliana. The diploid self-fertile plant Lotus japonicus has been identified and exploited as model legume by the research community and is now widely used in genetic and molecular studies. Methodologies have been developed over the last nine years allowing the routinely production of transformants. Since in planta transformation procedures have not been described yet in Lotus japonicus, in vitro procedures still represent the only available tools to obtain transgenic Lotus plants. The aim of this article is to review several protocols for both Agrobacterium tumefaciens and rhizogenes mediated in vitro transformation.*

#### A. TUMEFACIENS-MEDIATED *IN VITRO* TRANSFORMATION

##### Introduction

The essential requirements in a gene transfer system for production of transgenic plants are: (1) availability of a target tissue including cells competent for plant regeneration, (2) a method to introduce DNA into those regenerable cells, and (3) a procedure to select and regenerate transformed plants at a satisfactory frequency. Regeneration procedures can be based on direct or indirect somatic embryogenesis. In *L. japonicus*, the attempting to obtain transgenic plants via direct somatic embryogenesis have been unsuccessful and therefore several procedures involving first a dedifferentiation or callus induction step followed by the organogenesis phases have been set up (Handberg and Stougaard 1992; Stiller *et al.* 1997; Martirani *et al.* 1999; Lohar *et al.* 2001). Organogenesis *in vitro* depends on the

application of exogenous phytohormones, in particular auxin and cytokinin, and on the ability of the tissue to respond to these phytohormones changes during culture. In general, three phases of organogenesis are recognisable, based on temporal requirement for a specific balance of phytohormones in the control of organogenesis. In the first phase, cells in the explants acquire “competence” which is defined as the ability to respond to hormonal signals of organ induction. The process of acquisition of organogenic competence is referred to as “dedifferentiation.” The competent cells in cultured explants are canalised and determined for specific organ formation under the influence of the phytohormone balance through the second phase. Then the morphogenesis proceeds independently of the exogenously supplied phytohormones during the third phase.

The practical requirements to be satisfied in a transformation regeneration procedure are:

- Ready availability of the target tissue. Possibility to maintain a continuous supply of explants as starting material for transformation
- High efficiency, economy and reproducibility, to readily produce many independent transformants for testing
- Technical simplicity, involving a minimum of demanding or inherently variable manipulations
- Unequivocal selection or efficient screening to recover transgenic plants from transformed cells
- Minimum time in tissue culture, to reduce associated costs and avoid undesired somatoclonal variation
- Stable, uniform (nonchimeric) transformants for vegetatively propagated species
- Simple integration pattern and low copy number of introduced genes, to minimise the probability of undesired gene disruption at insertion sites, or multicopy-associated transgene silencing
- Stable expression of introduced genes in the pattern expected from the chosen control sequences.

In this article, we’ll review several transformation procedures that satisfy this list of requirements.

## **Root explants as starting material**

### **Materials**

All regeneration media are based on Gamborg's B5 medium (Gamborg, 1970) and contain B5 basal salts (Sigma, cat. # G-5768), B5 vitamins (Sigma, cat. # G-1019), 3% sucrose, pH 5.7 and 1 % agar (Sigma, cat. # A-7921) if not otherwise specified. Media differ in hormone and antibiotics composition:

- Callus inducing medium (CIM): 3 mg/L<sup>-1</sup> indoleacetic acid (IAA); 0.15 mg/L<sup>-1</sup> 2,4-dichlorophenoxyacetic acid (2,4-D); 0.6 mg/L<sup>-1</sup> benzyladenine

A.J. Márquez (Editorial Director). 2005. *Lotus japonicus* handbook. pp. 251-259.  
<http://www.springer.com/life+sci/plant+sciences/book/978-1-4020-3734-4>

(BA); 0.3 mg/L<sup>-1</sup> isopentenyladenine (IPA).

- Shoot inducing medium (SIM): 0.2 mg/L<sup>-1</sup> benzyladenine (BA) plus 10 mM NH<sub>4</sub><sup>+</sup>SO<sub>4</sub>.
- Shoot elongation medium (SEM): B5 medium, 1% Sucrose.
- Root inducing medium (RIM): B5/2 medium; 0.1 mg/L<sup>-1</sup> naphthaleneacetic acid (NAA).

Preparation of hormone and antibiotics stock solutions followed manufacturers instructions. Petri dishes and plant containers (Sigma, cat. # P1552) were used for cultivation of explants.

21 days transfer intervals are used during selection of transformed tissue.

### Transformation/regeneration procedure

The description of this procedure has been adapted from Lombardi *et al.* 2002. *Lotus japonicus*, GIFU ecotype F9 seeds are sterilised (20 min in 25% commercial bleach [1 % hypochlorite], 0.1 % Triton) and washed 6 times in sterile water. After resting overnight at 4°C, seeds are spread on the surface of 1% agar plates and left o/n, cap side down at 4°C. Plants are then transferred in the culture cabinet (23°C, 16 h photoperiod). Young seedlings (6 days old) are transferred on B5 standard medium plates (15/140 mm Petri dishes) placed vertically. The plants are allowed to grow for 3 weeks. The freshly harvested roots are spread evenly on CIM such that the entire root system is in contact with the medium. During this incubation, a visible increase of the root thickness is observed. Usually roots from one plate of plants are divided onto two CIM plates. Plants can be also stored at 4°C for two months and pre-incubated at 23°C for two days before to proceed with incubation of the excised roots on CIM medium.

For co-cultivation, the roots, which have been incubated on CIM for five days, are dipped in the appropriate *Agrobacterium* culture (about 5 X 10<sup>9</sup> cells/ml) and wounded by cutting into 0.5-cm pieces and by squeezing firmly with forceps (about 16 explants per plant and 30 explants/100 mm Petri dish). The root pieces are then blotted quickly on sterile paper and transferred on fresh CIM medium plates (about 40/100 mm Petri dishes). After two days of co-cultivation, the bacterial mass is removed by rinsing the explants in fresh B5 medium, blotting on sterile paper and transfer on CIM medium plus 200 mg/L<sup>-1</sup> cefotaxime.

After two days, the explants are transferred onto CIM medium containing 200 mg/L<sup>-1</sup> cefotaxime and 15 mg/L<sup>-1</sup> hygromycin (4 days after infection).

After three to four weeks, the putative transformants are scored for resistance and green sectors (0.2-0.4 cm) are isolated and transferred on SIM containing 200 mg/L<sup>-1</sup> cefotaxime and 15 mg/L<sup>-1</sup> hygromycin plus 10mM NH<sub>4</sub><sup>+</sup>SO<sub>4</sub>. Green calli are compact and easily separable from root segments (Figure 1). The average number of *Hyg*<sup>R</sup> calli obtained per explant is 1.23 and 70-90% of segments provided transgenic calli (about 22 per plant). The time of exposure of the explants to the CIM could be either further shortened by moving the explants to the shoot inducing medium (SIM) before the appearing of the green calli (3 weeks). This short incubation does not affect the frequency of callus formation and the *Hpt*<sup>R</sup> green calli

showing up on the SIM maintain a good cell division capacity by reaching a considerable size before to start shoot differentiation.



Figure 1. On the left, root explants not infected with *Agrobacterium* after an incubation of five weeks on CIM plus 15 mg/L<sup>-1</sup> hygromycin. On the right, root explants with Hyg<sup>R</sup> green calli obtained after an incubation of four weeks on CIM plus 15 mg/L<sup>-1</sup> hygromycin.

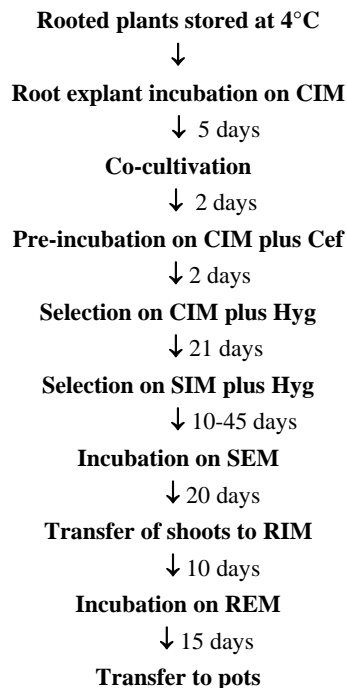


Figure 2. Flowchart of the root explant transformation regeneration procedure.

A.J. Márquez (Editorial Director). 2005. *Lotus japonicus* handbook. pp. 251-259.  
<http://www.springer.com/life+sci/plant+sciences/book/978-1-4020-3734-4>

Calli with small shoots emerging are then transferred to SEM. Further elongation is achieved after transfer the shoots on B5 medium without hormones and  $\text{NH}_4^+\text{SO}_4$ . Root induction is achieved on 2-4 cm long shoots by a ten days subculture on RIM and further subculture on B5/2 medium without hormones. The rooted  $T_1$  plants are healthy and produced normal flowers and seeds. A schematic description of the hypocotyl procedure is shown in Figure 2.

## Hypocotyl as starting material

### Materials

All regeneration media are based on Gamborg's B5 medium and contain B5 basal salts (Sigma, cat. # G-5768), B5 vitamins (Sigma, cat. # G-1019), 2% sucrose, 20  $\text{mg/L}^{-1}$  Hygromycin, 5  $\text{mg/L}^{-1}$  G418, 15  $\text{mg/L}^{-1}$  PPT, and gelrite 0.2% (unless otherwise specified), pH 5.7. Media differ in hormone and antibiotics composition.

- **Co-cultivation medium:** 10 fold diluted B5 liquid medium without sucrose, BA 0.5  $\text{mg/L}^{-1}$ , NAA 0.05  $\text{mg/L}^{-1}$
- **Callus-inducing medium:** (CIM): BA 0.5  $\text{mg/L}^{-1}$ , NAA 0.05  $\text{mg/L}^{-1}$  cefotaxime 300  $\text{mg/L}^{-1}$
- **Shoot-inducing medium:** (SIM): BA 0.2  $\text{mg/L}^{-1}$ , 10mM  $\text{NH}_4\text{SO}_4$ , cefotaxime 300  $\text{mg/L}^{-1}$
- **Shoot-growth medium:** (SGM): BA 0.2  $\text{mg/L}^{-1}$ , cefotaxime 300  $\text{mg/L}^{-1}$
- **Shoot-elongation medium:** (SEM): no hormones
- **Root-inducing medium:** (RIM): B5/2 medium, NAA 0.5  $\text{mg/L}^{-1}$ , 0.4% Gelrite
- **Root-elongation medium:** (REM): B5/2 medium, 0.4% Gelrite.

### Transformation-regeneration procedure

The description of this procedure has been adapted from Handberg and Stougaard 1992; Stiller *et al.* 1997. Sterilised seeds (20 min. in 2% hypochlorite, 0.01% Tween 20, several washing with sterile water) are germinated 7 days on water-soaked filter paper in darkness under a 23°/20°C (16/8 hours) temperature regime. Sterile hypocotyls are cut transversally or longitudinally in 2-3 pieces on filter paper soaked with culture of *Agrobacterium*. After cutting, explants are transferred immediately onto a pile of filter paper soaked with co-cultivation medium for 7 days in the dark. To provide mixture, additional medium is added next to the filter paper. After 7 days, the top filter paper with the hypocotyl pieces is moved to CIM plus cefotaxime.

Selection of transformed cells is started 5 days later by moving individual hypocotyl pieces on CIM plus G418, hygromycin or PPT. Green calli are obtained in average after a 4-week incubation. Transformed tissues are maintained on selective plates until the appropriate size for transfer to shoot induction plates is reached. Shoot induction is achieved in 3-6 weeks. Explants are transferred to fresh medium every week during selection, shoot induction and elongation stages. Well-grown shoots

A.J. Márquez (Editorial Director). 2005. *Lotus japonicus* handbook. pp. 251-259.  
<http://www.springer.com/life+sci/plant+sciences/book/978-1-4020-3734-4>

are cut off and vertically inserted into RIM and incubated for a week. Then, root elongation is performed on REM for 3-4 weeks. A schematic description of the hypocotyl procedure is shown in Figure 3.

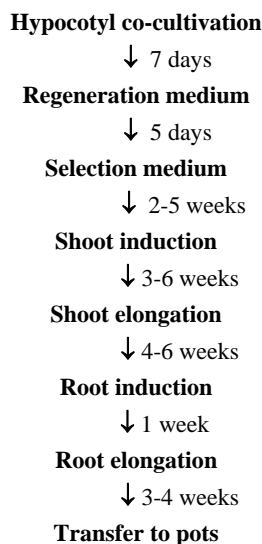


Figure 3. Flowchart of the hypocotyl explant transformation regeneration procedure.

## **A RHIZOGENES-MEDIATED TRANSFORMATION AND NODULATION OF COMPOSITE PLANTS**

### **Introduction**

The establishment of hairy root transformation and nodulation procedures in several legumes, including *Lotus corniculatus*, *Lotus japonicus*, *Vicia hirsuta*, and soybean, has enabled the studies on regulation and function of genes involved in nodule organogenesis and functioning (nodulins) in different genetic backgrounds. This experimental system represents the so-called short-cut system that allows the analysis of the promoter activity of nodulins as well as other genes involved in different organogenesis programs that take place in the root system. The transgenic root system obtained after the *A. rhizogenes* infection is suitable for the analysis of the promoter activity by exploiting appropriate reporter fusions and can be obtained in about one month hence avoiding the time consuming procedure for plant regeneration.

### **Hypocotyl infection**

This procedure has been adapted from Stiller *et al.*, 1997. Sterilised seeds are germinated on a wet filter paper in the dark, 24°C for 2 days. Germinated seeds are

A.J. Márquez (Editorial Director). 2005. *Lotus japonicus* handbook. pp. 251-259.  
<http://www.springer.com/life+sci/plant+sciences/book/978-1-4020-3734-4>

transferred on the top of agar slopes in square Petri dishes (1/2 X B5 medium, no sucrose, 1.2% agar) with root tips dipped into the medium. The seedlings are incubated in a growth chamber at vertical position (16/8 hrs day/night cycle). Three days later plantlets with unfolded cotyledons are inoculated in the hypocotyl area with a syringe and 1-2 drops of *A. rhizogenes* inoculum (late logarithmic phase; growth in YEB medium) is applied at the wound. Approximately 3 days post infection wounded hypocotyl areas become swollen and 2-3 days later, small callus starts to grow from the wound. The first hairy roots appear about 14 days post-infection and 1-3 cm long hairy roots develop in 3-4 weeks.

Composite plants (wild type shoot over a transgenic root system) with 1-2 cm long hairy roots are transferred on 1/2 X B5 medium to promote seedling growth and the primary root is cut off. Four days later plants are transferred on no Nitrogen source medium slopes (B&D, Jenssen) and inoculated by applying a drop of *M. loti* inoculum on newly grown root tips.

### Root infection

This procedure has been adapted from Martirani *et al.*, 1999. Surface sterilised seeds are germinated and grown vertically on 1% agar plates. Six days-old seedlings are transferred on NLN medium (Duchefa) containing 1% sucrose. After two days, the primary roots of the seedlings can be cut at different distance from the root tip. The freshly cut surface is inoculated with *A. rhizogenes* strains grown overnight in LB medium. After two days of co-cultivation, the seedlings are washed and blotted on sterile paper before to be transferred on B5 medium supplemented with 1% sucrose and 200 mg/L cefotaxime. A callus is visible at the wound site 5 days after infection. After 10 days, microcalli with emerging hairy roots appear clearly at the wound sites (Figure 4).



Figure 4. Plant infected with *A. rhizogenes* showing a well-developed transgenic root system.

The composite plants with hairy roots are maintained on B5 medium for at least two weeks until a massive root system is obtained. During this incubation, the roots that arise above the wounded site are eliminated with scissors. Analysis of the root system obtained after infection with a *A. rhizogenes* strain equipped with a T-DNA

A.J. Márquez (Editorial Director). 2005. *Lotus japonicus* handbook. pp. 251-259.  
<http://www.springer.com/life+sci/plant+sciences/book/978-1-4020-3734-4>

vector carrying a 35S-*gusA* cassette indicates that in average 80% of the plants are transformed and about 70% of the roots show GUS activity (Figure 5), indicating a high frequency of co-transformation of the *gusA* and pRi-born T-DNAs.

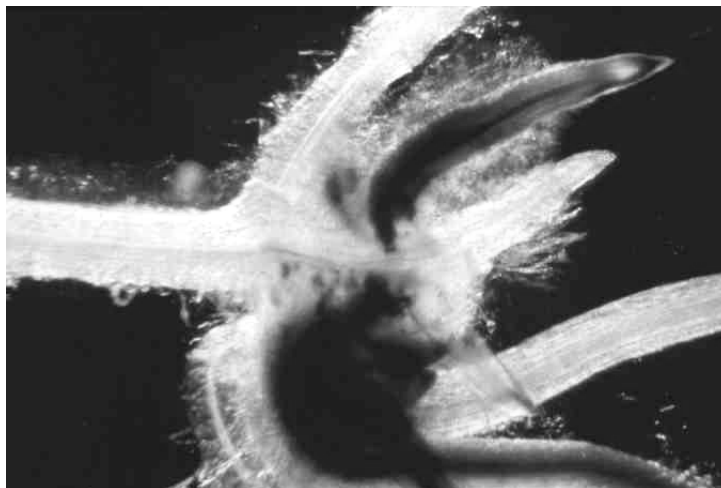


Figure 5. GUS staining obtained in hairy roots co-transformed with the T-DNA vector carrying the 35S-*gusA* cassette. See colour plate 4 (G) for this figure in colour.

Composite plants are transferred to slanted fresh NLN medium 1% agar. Sterile filter paper is first applied over the taller part of the slope where the root system will be located. This is to avoid growth of the roots into the agar. Petri dishes are located vertically in a growth cabinet at 22°C with a 16-hrs photoperiod. The roots are maintained in the dark by using aluminium foil. Each primary meristem is inoculated with a drop of *Mesorhizobium loti* NZP2235 suspension containing about 10<sup>6</sup> bacteria.

## COMMENTS

In this article, we describe several Agrobacterium-mediated transformation procedures adapted from different papers. These protocols differ for some features such as the target tissue used as starting material, the infection procedures, and regeneration media. In particular, the efficiency of transformation in the *A. tumefaciens*-mediated procedure is comparable (about 1.5 transformants per explant) whereas the incubation time on CIM medium differs significantly (one month shorter in the root explant procedure). On the other hand, the use of the mutagen 2,4-D is not provided in the CIM medium of the hypocotyl transformation procedure. Furthermore, an important point of the root explant procedure is the possibility to use plants stored in the cold room for long time as source of the explant hence providing a possibility to have a continuous supply of starting material for transformation.

*In planta* transformation procedures like those published for *A. thaliana* (Bechtold *et*

A.J. Márquez (Editorial Director). 2005. *Lotus japonicus* handbook. pp. 251-259.  
<http://www.springer.com/life+sci/plant+sciences/book/978-1-4020-3734-4>

*al* 1993; Clough and Bent 1998) and, more recently, for *Medicago truncatula* (Trieu *et al.* 2000) represent the next challenge for the *L. japonicus* community. However, the techniques described here ensure high reproducibility and efficiency with reasonably quick regeneration times (about 4-5 months to obtain primary transformants to be transferred in soil for *A. tumefaciens*).

## REFERENCES

- Bechtold N, Ellis J, and Pelletier G. (1993) **In planta *Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants.** *Comptes Rendus de l'Academie des Sciences, Serie III-Sciences de la Vie* 316, 1194-1199.
- Clough SJ and Bent AF. (1998) **Floral dip. a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*.** *The Plant Journal* 16, 735-743.
- Lohar D, Schuller K, Buzas DM, Gresshoff PM, and Stiller J. (2001) **Transformation of *Lotus japonicus* using the herbicide resistance bar gene as selectable marker.** *Journal of Experimental Botany* 52, 1697-1702.
- Martirani L, Stiller J, Mirabella R, Alfano F, Lamberti A, Radutoiu SE, Iaccarino M, Gresshoff PM, and Chiurazzi M. (1999) **Establishment of a T-DNA Tagging Program in the Model Legume *Lotus Japonicus*.** Expression patterns, activation frequencies and potential for insertional mutagenesis. *Molecular Plant-Microbe Interactions* 12, 275-284.
- Handberg K and Stougaard J. (1992) ***Lotus japonicus*, an autogamous, diploid legume species for classical and molecular genetics.** *The Plant Journal* 2, 487-496.
- Stiller J, Martirani L, Tuppal S, Chian R, Chiurazzi M, and Gresshoff PM. (1997) **High frequency transformation and regeneration of transgenic plants in the model legume *Lotus japonicus*.** *Journal of Experimental Botany* 48, 1357-1365.
- Trieu AT, Burleigh SH, Cardailsky IV, Maldonado-Mendoza IE, Versaw WK, Bleylock LA, Shin H, Chiou TJ, Katagi H, Dewbre GR, Weigel D, and Harrison MJ. (2000) **Transformation of *Medicago truncatula* via infiltration of seedlings or flowering plants with *Agrobacterium*.** *The Plant Journal* 22, 531-541.