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# Chapter 6.2

## INDUCTION OF HAIRY ROOTS FOR SYMBIOTIC GENE EXPRESSION STUDIES

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*The model legume* Lotus japonicus *can be transformed and regenerated efficiently with* Agrobacterium tumefaciens *or* A. rhizogenes. *However, it takes between 8 to 12 months to obtain seeds of transgenic plants. We therefore developed a rapid and efficient transformation protocol using* A. rhizogenes *to induce transgenic hairy roots that can be inoculated with* Mesorhizobium loti *2 weeks after transformation. The first nodules emerge 8 to 10 days after inoculation, as on the roots of wild type Lotus plants and expression of plant genes involved in any step of nodulation can be completed within two months after the start of a transformation-nodulation experiment. A large number of seedlings can be tranformed in one experiment, allowing addressing of a number of variables in one single tranformation-nodulation experiment.*

## INTRODUCTION

*Lotus japonicus* (Regel) Larsen has been proposed as a model plant to study the symbiosis between leguminous plants and rhizobia using classical and molecular genetics because of its small genome size, short life cycle and high seed yield (Handberg and Stougaard 1992, Jiang and Gresshoff 1997). Moreover, *L japonicus* can be regenerated after transformation of hypocotyls segments by *Agrobacterium tumefaciens* (Handberg and Stougaard 1992, Handberg et al 1994, Oger et al 1996, Stiller et al 1997, Quaedvlieg et al 1998) transverse cotyledon, or root segments (Aoki et al 2002; Lombari et al 2003). Highly efficient transformation and regeneration of *L japonicus* plants using *A. rhizogenes* has also been obtained

(Stiller et al 1997; Martirani et al 1999). In these protocols, tissue culture periods ranging from 4 to 9 months before transfer of the transgenic plantlets to soil are reported, with seed setting beginning 3-4 months later.

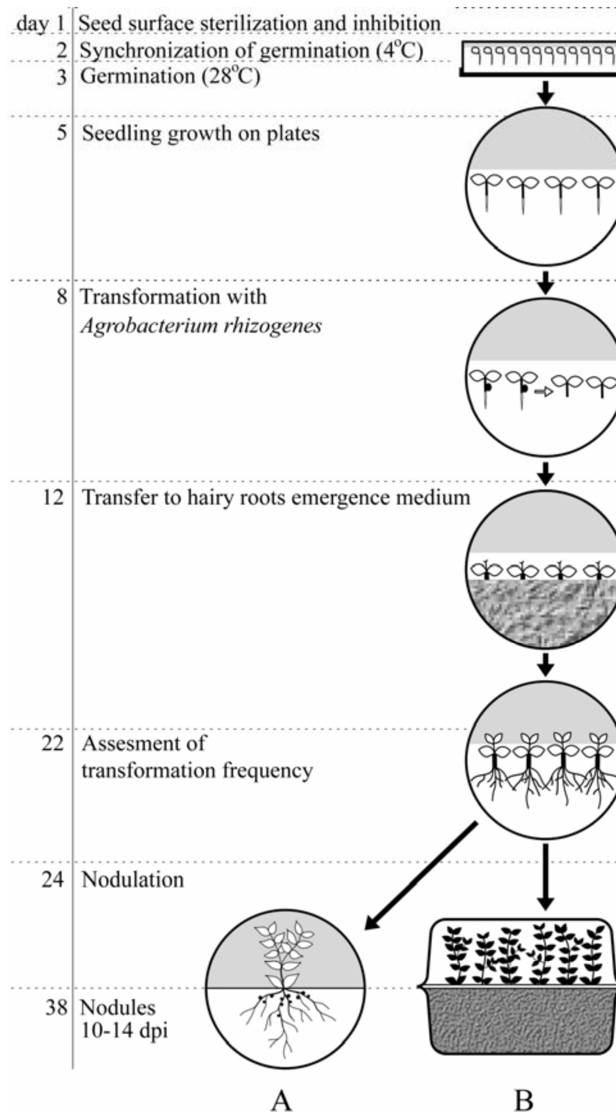


*Figure 1. Flow chart of a hairy roots induction-nodulation experiment on L japonicus seedlings. The left columm gives the timing where each step is carried out starting from seeds to the observation of the first root nodules by eye inspection on plants grown on agar-plates (A) or in nodulation boxes (B).*

Although useful for the production of transgenic plants, *A. tumefaciens*-based transformation-regeneration protocols are not practical when the aim is to rapidly

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test gene expression in Lotus roots during nodulation. Therefore we set up a faster and more efficient transformation system for *L japonicus* ecotype 'Gifu' using *A. rhizogenes* carrying the genes of interest in binary vectors. Nodulation assays on transformed hairy roots can be set up 2 weeks after transformation and nodulation kinetics proceed as in wild type plants. Nodules are pink and able to fix nitrogen, as shown by acetylene reduction assay. Because large number of plants can be transformed (per experiment, 200 to 300 seedlings can be comfortably transformed by one experienced worker), statistical analysis of the expression of the gene or genes of interest can be easily achieved.

The protocol we describe in this chapter is based on the induction of hairy roots on white clover seedlings (Díaz et al., 1989) and can be divided into four steps: seed surface sterilization and germination, co-cultivation and transformation, hairy root emergence, and nodulation of transformed plants. The approximate timing of each step appears in the flow chart depicted in Figure 1.

## MATERIALS AND INSTRUMENTATION

### Solutions for seed surface sterilization

Sterilised, deionized water suitable for tissue culture (Millipore) is used in all solutions. For surface sterilisation, we use a 4-5% natrium hypochlorite solution that can be purchase in local supermarkets. It does not contain detergents.

### Bacterial Media

#### Solid medium for Agrobacterium rhizogenes

- Bactotryptone (DIFCO) 50.0 g
- Yeast extract (DIFCO)" 5.0 g
- NaCl 8.0 g
- $MgSO_4 \cdot 7H_2O$ 12.3 g
- 1 M Tris 1 mL

Adjust pH to 6.4 to 6.8, bring to 1 L and add Select Agar (Gibco BRL, Difco Lab. Paisley, Scotland) to 1.8%. Sterilize for 20 min at 120$^oC$.

#### Solid medium for *Mesorhizobium loti* (YMB)

- Mannitol 10.0 g
- Yeast extract (DIFCO) 0.4 g
- $MgSO_4 \cdot 7H_20$ 0.2 g
- NaCl 0.1 g
- $K_2HPO_4 \cdot 3H_2O$ (or $K_2HPO_4$) 0.65 g (or 0.5 g)

Adjust pH to 7 using 2 M HCL, bring to 1 L, and add Select Agar (Gibco BRL, Difco Lab. Paisley, Scotland) to 1.8% and sterilize for 20 min at 120$^oC$.

## Medium for germination of *L japonicus* seeds (Jensen)

| | |
|---|---|
| • $CaHPO_4$ | 1.0 g |
| • $K_2HPO_4$ (or $K_2HPO_4{\cdot}3H_20$) | 0.2 g (0.26 g) |
| • $MgSO_4{\cdot}7H_20$ | 0.2 g |
| • NaCl0 | 2 g |
| • $FeCl_3{\cdot}H_20$ | 0.1 g |
| • Trace elements solution | 12.5 mL |

Bring to 1 L. To solidify, add 9 g Daishin agar (Daiichir Seed Company, Japan). Sterilize for 30 min at 110$^o$C. (Liquid Jensen medium can be sterilized for 20 min at 120$^o$C and stored at room temperature for up to 5 months. Formation of a precipitate is a normal feature of this medium (Van Brussel et al., 1982)).

**Trace elements solution**

| | |
|---|---|
| • $CuSO_4{\cdot}5\ H_20$ | 35.4 mg |
| • $MnSO_4{\cdot}4H_20$ (or $MnSO_4{\cdot}H_20$) | 609 mg (or 462 mg) |
| • Zn $SO_4{\cdot}7H_20$ | 97.4 mg |
| • $H_3BO_3$ | 1269 mg |
| • $NaMoO_4{\cdot}2H_2O$ | 398 mg |

Bring to 1 L Do not sterilize this solution. Keep in frozen in aliquots.

## Medium for co-cultivation and nodulation of transformed *L japonicus* seedlings

Prepare solidified Jensen as described. Allow to cool down to 65$^o$C and add (per L) 1.5 mL of a sterilized 1 M $NH_4NO_3$ solution.

## Medium for hairy roots emergence (HRE)

This medium is based on SH medium (Schenk and Hildebrandt, 1971) and contains 300 µg cefotaxime per mL.

- 20X concentrated SH-A salts
- 20X concentrated UM-C vitamins
- 10 g sucrose
- 3 mL 1 M MES

To prepare a litre of medium, combine 700 mL deionized, tissue culture grade water with the above components. Rinse the tubes containing the stocks 2-3 times with water and add to the cylinder. Adjust pH to 5.8 using 1 M KOH. Adjust volume to1 litre. For solidified medium, add Daichin agar to 0.9%. Sterilize for 30 min at 110$^o$C. Add 3mL cefotaxime stock solution after cooling the medium to 65$^o$C and

pour plates immediately. Allow the plates to dry for 45 minutes before storage. Liquid HRE sterilized medium can be kept frozen in aliquots for 3 months. Allow the medium to come to RT before adding cefotaxime (see below) and use immediately.

**Concentrated (20X) SH-A salts**

- $KNO_3$ 50 g
- $MgSO_4.7H_2O$ 8 g
- $NH_4H_2PO_4$ 6 g
- $CaCl_2.2H_2O$ 4 g
- $MnSO_4.4H_2O$ 0.2 g
- $H_3BO_3$ 0.1 g
- $ZnSO_4.7H_2O$ 0.02 g
- KI 0.02 g
- $CuSO_4.5H_2O$ 0.004 g
- $NaMoO_4.2H_2O$ 0.002 g
- $CoCl_2.6H_2O$ 0.002 g
- $FeSO_4.7H_2O$ 0.3 g
- NaEDTA(TiTriplex) 0.4 g

Use deionized tissue culture grade water to bring to a final volume of 1 L For $FeSO_47H_2O$, prepare a solution in 100 mL warm water. For NaEDTA (TiTriplex), prepare a solution in 100 mL warm water. Dissolve the other salts in 700 mL water. Add the $FeSO_4.7H_2O$ and NaEDTA solutions and adjust the volume to 1 L Store 50 mL aliquots at -20$^o$C until use.

**Concentrated (20X) UM-C vitamins**

- Myoinositol 2.0 $gL^{-1}$
- nicotinic acid 0.1 $gL^{-1}$
- Pyridoxine HCl (Vitamin B6) 0.2 $gL^{-1}$
- thiamine HCl 0.2 $gL^{-1}$
- Glycine 0.04 $gL^{-1}$

Use deionized tissue culture grade water. Dissolve in 900 ml $H_2O$, adjust volume to 1L, divide in aliquots of 50 mL and keep at –20$^o$C until use.

**MES (2-[N-Morpholino]ethane-sulfonic acid), 1M stock solution**

To prepare 150 mL of a 1 M solution weight 29.28 g MES (Sigma, Cell Culture) and add to 125 mL water. Adjust to pH 5.8 with a 10 M KOH solution. Adjust MES volume to 150 mL, divide in aliquots of 6 mL and store at –20$^o$C until use. Before

adding to HRE medium, warm the MES solution to avoid precipitates.

**Cefotaxime sodium** (Duchefa, Haarlem, The Netherlands)

For a 10 mL stock solution containing 100 mg $mL^{-1}$: dissolve 1 g cefotaxime in 9 mL water and filter-sterilize it. Keep at 4°C in the dark, use within 2 months.

## Preparation of growth plates

### Germination plates

Germination plates are 100X25 mm (Labtec, Nalge Nunc Int. Aurora, Ohio, USA, article LX4031). They contain 25 mL Jensen medium, solidified with 0.9% Daichin agar. After pouring and drying for 45 min, two filter papers (cut following the contour of the plate and sterilized as described in *VI*.5) are placed on the inner surface of the lids. Closed plates, wrapped in plastic, can be stored for 2-3 months at room temperature.

### Transformation plates

Transformation plates (94X16 mm, C.A. Greiner & Shöne Ges. m.b. H., Kremsmünster, Austria, article 633102) contain 30-35 mL solidified Jensen medium (0.9% Daichin agar) supplemented with 1.5 mM $NH_4NO_3$. After drying for 45 min., semicircles of sterilized filter paper, 5.5 cm in diameter (see Preparation of growth plates, Filter paper), are placed on top of the agar, to cover half the plate. Closed plates, wrapped in plastic, can be stored (filter side up) up to 2 months at RT.

### HRE (hairy roots emergence) plates

HRE (hairy roots emergence) plates (94X16 mm, C.A. Greiner & Shöne Ges. m.b. H., Kremsmünster, Austria, article 633102) contain solidified HRE medium supplemented with 300 μg $mL^{-1}$ cefotaxime. Plates can be stored (medium side up) up to one week wrapped in plastic in the dark at 4°C.

### Nodulation plates

Nodulation plates, (150X20 mm, from Sarstedt Inc., Newton, Ohio, USA, article 82.1184), contain 60-70mL Jensen supplemented with 1.5 mM $NH_4NO_3$. After drying for 45 min, a semicircle of sterilized filter paper 8.5 cm in diameter (see Preparation of growth plates, Filter paper) is placed on top of the agar, as to cover half a plate. Closed plates, wrapped in plastic, can be stored (filter side up) for 2 months at RT.

## Filter paper

Filter paper (Schleicher &Schuell sheets, article 334580) is used to maintain a thin layer of nutrient solution in direct contact with roots. Semicircles (5.5 cm in diameter for transformation and root hair emergence plates, and 8.5 cm for nodulation plates, respectively) are cut following the contour of the base of the plates and spread as the tiles of a roof on a sheet of aluminium foil, in such a way

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that they can be individually pick up with a forceps without compromising sterility. Filter papers, wrapped in aluminium foil are sterilized for one h at 120°C.

## Plastic strips

Plastic strips (PVC) used in nodulation plates are 140X18 mm, 1 mm in width. They have a split 7 mm in length and 1.5 mm in width located at the center of the strip. The split should be wide enough to let the stem of a Lotus plant go through it without damage. The strip is inserted into the agar so far that when the lid is placed and the plate stands in vertical position, it prevents direct light reaching the roots. Plastic strips are kept in 70% ethanol and are air-dry (down flow cabinet) before placing them in nodulation plates.

## Parafilm

It is important to use Parafilm™ correctly in order to avoid leaking of liquid contaminated with Agrobacterium. To seal transformation and HRE plates unroll Parafilm and cut 7X5 cm strips. For nodulation plates, obtain 14X5 cm strips of Parafilm. Plates are sealed by pressing longitudinally stretched Parafilm strips on ¾ of each plate, allowing gases to diffuse above cotyledonary and real leaves. To avoid leakage due to tearing, Parafilm strips should be slightly stretched, just to obtain firm attachment to the plates. Placing a piece of black cardboard between the plates also helps to prevent tearing.

## Nodulation boxes

The bottom part of tissue culture boxes (bottom RA60, lids RDA145/60, sold by ECOLINE BVBA, Pierkenstraat 82, B-9620 Zottegem, Belgium, teL +32 09 360 97 10, Fax +32 054 300 696) are filled with wet clay pellets to 4-5 cm in height. Clay pellets (the type used in hydroponic plant culture) are 2-4 mm in diameter and can be purchased from Hydro Jongkind BV, Alsmeer, Holland, htpp://www.jongkind-grond.nl/ or from Handelsvertreter Deutschland und Österreich, Herget's Blumenzwiebel, Rosenstrasse 28, D-82024 Taufkirchen, Germany; Tel. +49 896 124 706, Fax +49 896 125 128. To sterilize, pellets are placed in pyrex oven dishes, rinsed several times with deionized water, and baked overnight in open dishes in an oven at 180°C. The lids of the pyrex dishes (baked separately) are placed as soon as the glass is cold and the oven is opened. Securing the lids with tape and wrapping the dishes in aluminum foil allows storage for long periods.

To prepare nodulation boxes, dried pellets are submerged in sterilized Jensen medium containing 1.5 mM $NH_4NO_3$ for 10 min. Excess of liquid is discarded and the bottom of the boxes is filled wit soaked clay particles using a large, previuously sterilized spoon. Closed, filled containers can be prepared advance and kept at RT for 2 weeks.

## Flow Cabinets

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### Laminar-flow cabinets

Laminar-flow cabinets are used to prepare plates and nodulation boxes, to surface sterilize seeds, and to spread germinated seedlings on transformation plates.

### Down-flow cabinets

Down-flow cabinets are used for transformation, transfer to HRE plates and nodulation plates, inoculation with rhizobia and addition of liquid nutrient media, if required.

The working surface in down-flow cabinets is protected from contamination with Agrobacterium by opening plates on a 1 L beaker placed on a piece of highly absorbent paper towel (25x15 cm). Towels are placed on aluminium foil and sterilized for 1 h at 120°C before use. If required, pieces of absorbent paper towel (2x2 cm), sterilized as above described, can be used to dry the edges of Petri dishes.

Items required for working in flow cabinets, such as glassware, forceps (straight, thin, light in weight and 24 cm long, Duchefa, Haarlem, The Netherlands), pipettes, spoons, etc., are previously sterilized before use.

## Growth Conditions

### Temperature, photoperiod, and light sources

Growth of seedlings before transformation, co-cultivation, hairy root emergence, and nodulation takes place at 20°C, with 16 h light and 8 h darkness. Transformation frequencies varying between 60 and 100% have been obtained by growing the plants with Philips TLD 30W/95, TLD 30W/33 and TLD30W/84 tubes at light intensities varying between 2000 to 6000 Lux.

### Stress during tissue culture conditions

In all experiments, we have observed that some seedlings can show signs of stress, which is apparent one to two weeks after growth in HRE plates. These signs are: anthocyanin formation in stems, yellowing of real leaves, retardation or failure to grow and production of new leaves, appearance of brown patches (possible tannins) on the root surface and retardation of root growth and lateral root formation. Plants that do not seem to be affected by stress (about 80% for plants grown under Philips TLD 30W/95 and 40-60% for plants grown under the other two light sources) are green, grow vigorously, and have whitish roots. Apparently, stress is not due to the composition of the growth media, or to transformation, because non-transformed control plants grown on different plant media and under the same light conditions as transformed plants, can show the same signs of stress. After a period of stress (about 10 days) 20-30% of the transformed stressed plants grown in HRE medium seem able to recover spontaneously. Stress can also be alleviated by shielding the roots from direct light and by transferring to fresh HRE medium.

### Stress during nodulation

Nodulation in agar plates also induces stress in transformed plants, even if the roots

are shielded from direct exposure to light. Nodulation in boxes, with roots growing in the dark in clay pellets, does not seem to induce stress. Moreover, stressed plants seem to recover quickly when transferred to these boxes.

## EXPERIMENTAL PROCEDURES

### Seed surface sterilization and germination

- Weigh seeds. Each seed is about 1 mg. An experienced operator can transform 300 to 400 seedlings in one experiment. Transfer to a 100 mL sterilized Erlenmeyer.
- Add enough concentrated $H_2SO_4$ to cover de seeds, let stand for 2 min.
- Add 50 mL water, mix quickly, and pour out immediately. Add more water, swirl seeds, and pour out. Repeat this step 5 times.
- Add a 4-5% sodium hypochlorite solution, just to cover the seeds, incubate for 10 min.
- Rinse as described in the third step.
- For imbibition: add enough water to obtain a 2-3 mm layer above the seeds and keep at room temperature in the dark. Seeds swell rapidly in the next 6 h, after this they can be placed at 4$^o$C. Alternatively, let imbibition proceed overnight at room temperature.
- Seeds are rinsed twice with water before pouring them on germination plates (see Preparation of growth plates, Germination plates). Place two sterilized filter papers on the inner surface of the lid of the plate and add water to wet them well, but do not overflow. Set imbibed seeds on the agar surface, close about ¾ of the plate with Parafilm, or completely around with leucopore.
- Synchronization of germination is achieved by placing the seeds at 4$^o$C in the dark for 24-48 h. Set the plates with seeds resting on the surface of the agar.
- For germination, plates are placed up-side down at 28$^o$C in the dark for 2 days, as to the roots will grow toward the wetted filter paper. A very high percentage of germination is obtained with this method. Roots show many root hairs and are uniformly about 1 cm in length.

### Infection and co-cultivation with Agrobacterium rhizogenes

- Pipette 1.5-2 mL Jensen medium containing 1.5 mM $NH_4NO_3$ onto transformation plates (see Preparation of growth plates, Transformation plates). Take care to use only clear Jensen solution, as the precipitate formed in the medium will interfere with microscopy.
- Exposure of the germination plates (up-side down) to light for 4-6 h triggers expansion of the cotyledons and facilitates removal of the seed

coat. After this period, germination plates are turned up side up and 5 mL Jensen is pipetted onto the seedlings to protect them against desiccation and to make seed coat removal an easier manipulation. In addition, small groups of seedlings can be easily separated and individual seedlings can be recovered without damage. Seedlings with roots similar in length are transferred to transformation plates. To synchronize the expansion of the cotyledons and to ensure straight-growing hypocotyls, the seed coat is removed by applying a slight pressure over the cotyledons. Seed coat removal results in vigorously growing seedlings that seem to help dealing with the stress caused by sectioning of the root and transformation. However, successful transformation with *A. rhizogenes* can be carried out without removal of the seed coat. Five or six undamaged seedlings are chosen and placed in a row, with the cotyledons on the filter paper, about 3 mm from the edge of the paper. Parafilm is used to seal about ¾ of the plates, leaving the part above the epicotyls open. Plates are placed vertically in a tray and transferred to the growth cabinet for 2 days.

- Have ready: plates of fully-grown *A. rhizogenes* LBA 1334 (Offringa et al., 1986) carrying the binary plasmids of interest (2 plates per strain, grown for 2-3 days at 28$^{o}$C on fresh selection plates, see Bacterial media, Solid medium for Agrobacterium). We use LBA 1334 because it transforms Lotus so efficiently, as we report in the comments.
- Infection: carefully, open transformation plates and streak *A. rhizogenes* on the hypocotyls, about 2-3 mm below the point of insertion of the cotyledons. The bacterium is taken directly from a selection plate with the closed tip of a glass Pasteur pipette. Antibiotics such as rifampicin, kanamycin, and spectinomycin do not seem to affect the plant. To synchronize the emergence of hairy roots, hypocotyls are sectioned with a scalpel in the middle of the zone streaked with Agrobacterium. Roots are discarded and if needed, Jensen is pipetted to keep a high degree of humidity into the plates. Sectioned plants will remain attached to the filter paper if cotyledons are placed on the paper' surface. Plates are sealed with Parafilm (as described in Preparation of growth plates, Parafilm) and placed in a growth tray. Note that no incubation time is required between application of Agrobacterium and sectioning of the roots. However, to increase the efficiency of this procedure, seedlings contained in 25-30 plates are usually infected before proceeding with sectioning.
- Co-cultivation: trays are placed in the growth cabinet for 5 days. At the end of this period, many seedlings present swellings at the wound site. In some cases, hairy tumours and emerging roots can be observed. It is very important to keep the plates very wet in order to allow this rapid response (when standing vertically, a little bit of liquid Jensen should be visible on the bottom of the plate). In some cases, emergence of the first real leaf could cause the seedling to fall down. Apparently, this does not affect transformation.

## Hairy root emergence

To obtain a high degree of humidity, 1.5-2 mL liquid HRE medium (hairy roots emergence Mat. and Inst. *V*) containing 300 mg $L^{-1}$ cefotaxime is added onto emergence plates (Mat. and Inst. *VI*.3).

On the transformation plates, seedlings that have fallen to the bottom of the plate are replaced in the row. Carefully, a new semicircle of filter paper is placed on the seedlings, in such a way as to cover the area of response to *Agrobacterium* infection. Sliding the bottom filter towards the centre of the plate facilitates the deposition of the cover filter. Cotyledons and apical meristems should not be covered with filter paper. The two filters, carrying the seedlings in between, are transferred to HRE plates. Plates, sealed with Parafilm as described, are placed in growth trays and are taken to the growth cabinet. Take care not to transfer seed coats or cut roots, as they will contribute to the development of infections.

To control seedling development and humidity of the plate during hairy root emergence, add liquid HRE medium containing cefotaxime if necessary. One week after transfer, about 80% of the seedlings will present more than two roots and 10-15% will have one or two roots. In addition, the first two real leaves will emerge and unfold. Seedlings will grow vigorously and new roots will emerge in the following days.

Transformation frequencies (for example, GUS activity) can be assayed 10-14 days after transfer to HRE plates.

If seedlings are going to be kept in tissue culture conditions for longer than 21 days, transfer transformed seedlings to fresh HRE plates. Pour 150X20 mm plates with HRE medium (follow instructions described in Preparation of growth plates, Nodulation plates). Place 4 or 5 seedlings per plate, cover roots with filter paper, and pipette 5-7 mL liquid HRE containing cefotaxime to keep humidity high. These larger plates allow space for seedling growth and development.

## Nodulation of transgenic hairy roots

Nodulation on agar plates: a transformed seedling is transferred to a nodulation plate (see Preparation of growth plates, Nodulation plates). The hairy roots tumor and emerged roots should be in contact with the filter paper. Rhizobia are applied to root tips and onto the hairy roots tumor directly from a plate, with the closed tip of a Pasteur pipette. Between 5-7 mL Jensen medium containing 1.5 mM $NH_4NO_3$ are added and a new filter paper is placed as to cover the roots and the tumor. To avoid direct exposure of the roots to the light, a plastic strip is inserted into the agar above the edges of the filter papers. About ¾ of the plate is sealed with a piece of Parafilm. A second piece of Parafilm is placed on top of the first one, to avoid leakage. The plates are placed vertically in a tray, between pieces of black cardboard (12X15 cm) and transferred to the growth cabinet.

Nodulation in tissue culture boxes (see Preparation of growth plates, Nodulation boxes) is achieved by planting inoculated roots in clay pellets contained in tissue culture boxes. 100% of innoculated plants nodulate and the average number of nodules counted 6 weeks after inoculation with *M. loti* strain R7A is 14 (most confined to one well-developed transformed root); wild type plants present 6-8 nodules at this time point. The roots of transformed seedlings on the HRE plates are

inoculated by applying rhizobia directly from a plate, as already described. A hole in the clay pellets is made with a tweezer and an inoculated plant is carefully transferred to the container. Clay pellets are then displaced to completely cover the roots. This procedude is facilitated if seedlings are inoculated 10 to 20 days after transfer to HRE plates, when the longest roots are between 3-5 cm in length. Usually, 6 to 8 plants can be placed in one container. The container is closed and transferred to the growth room.

## COMMENTS

### Flow chart

In this protocol, we have given the minimal time required for each step to be accomplished with success (70-100% transformation frequencies using different binary vectors, see table 1 below for details). The percent of roots showing GUS activity is consistent with the expected frequency of roots co-transformed with the gusA and pRi born T-DNAs. However, there are margins of variation in the timing of each step that do not affect transformation frequencies and facilitate carrying out some steps. For instance, imbibed seeds resting on the agar surface of the plate can be kept at 4$^{o}$C as long as the seeds do not dry out (about one month), as drying out dramatically decreases germination. In addition, germination proceeds well, although slower, at 25$^{o}$C with a 16h light/8h darkness photoperiod. Infection with *A. rhizogenes* can be performed within 2 to 5 days after transfer of seedlings to transformation plates, without compromising transformation frequencies. Note that transfer to plates containing HRE medium on time (4-6 days after infection with Agrobacterium) speeds up root emergency and decreases stress.

### Agrobacterium rhizogenes *LBA1334*

As can be seen in Table 1 (next section), this strain is very efficient in the transfer of DNA carried by the binary plasmids we tested into *L japonicus* cells. In addition, LBA1334 induces a very small tumour from which multiple cells are able to rapidly differentiate into roots (Figure 2), which can effectively be used for nodulation studies. This strain, a derivative of *A. tumefaciens* strain C58C9 with a chromosomal marker for rifampicin (Rif), carries the agropine-type pRi1855 with a spectinomycin resistance marker (Spc) in a segment that is not involved in virulence (Offringa et al., 1986). Binary plasmids can be efficiently crossed by triparental mating (Ditta et al, 1980) or electroporation (den Dulk-Ras et al., 1995). To prepare selection plates use LC medium (see Bacterial media, Solid media for Agrobacterium) containing Rif 20µgmL$^{-1}$, Spc 250µgmL$^{-1}$ and the bacterial selection marker encoded in the binary plasmid.

We have also found that LBA1334 is resistant to chloramphenicol. Binary vectors from the pPZP family (Hajdukiewicz et al., 1994) can be selected using kanamycin 100µgmL$^{-1}$ since neomycin phosphotransferase II is expressed under the control of 35S CaMV promoter, which is active in this strain.

We never keep Agrobacterium strains on plates; instead, we prepare a back-up stock

(kept at -80°C) and a working stock (kept at -80 or -20°C) that is used to inoculate freshly poured plates containing the required antibiotics 4 or 5 days before transformation is planned. LBA1334 from stocks grows well within 48 h at 26-28°C. A second set of freshly poured plates containing the required antibiotics is then used to grow Agrobacterium for transformation. Stocks are obtained from fully grown plates prepared as described above by suspending the bacteria in 3 mL previously sterilized 0.6% peptone-15% glycerol before freezing.

## Frequency of transformation

The following table summarizes transformation frequencies of various binary vectors, based on histological staining of the CaMV35S-*gus:intron* reporter gene using 5-bromo-4chloro-3-indolyl-β-glucoronic acid as substrate, performed 7 to 20 days after transfer to HRE medium. Seedlings can present 2 to 10 rapidly growing roots, and many loci of transformed cells that are not yet differentiated into (growing) roots (see also Figure 2).

| **Binary vector** | **Percent of plants with transformed roots**[a] | **Percent of GUS-expressing roots**[1] |
|---|---|---|
| pBin19-gusAINT[b] | 85±15 | 62±8 |
| pPZP-based[c] | 90±10 | 75±5 |
| pCAMBIA1301[d] | 78±5 | 50±10 |

*Table 1. Transformation frequencies of L japonicus by LBA 1334-carrying various binary vectors with the gus:intron reporter gene driven by the CaMV 35S promoter. Data are the mean of at least three independent transformation experiments. GUS assay was performed in lots of 20 to 50 plants. [a]Only emerged roots were considered when calculating the % of transformed roots at the time of staining. [b]Vancanneyt et al (1990). [c]Hajdukiewicz et al (1994). [d]Robert s et al (1997).*

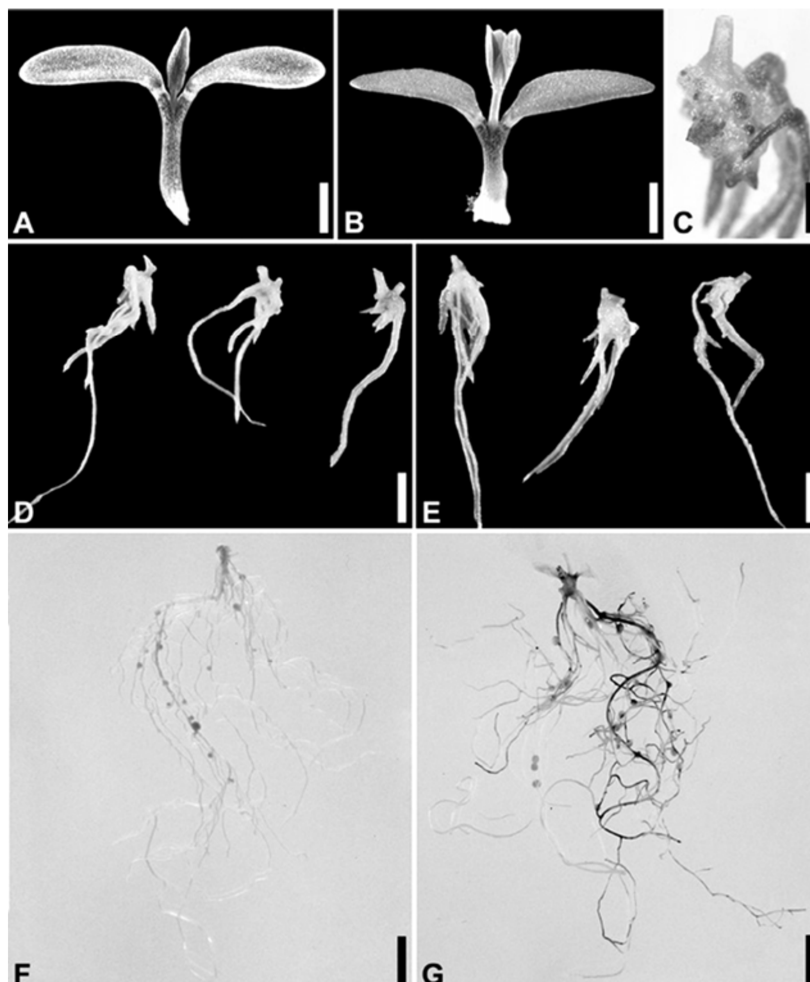


*Figure 2. Hairy roots induced by A. rhizogenes LBA 1334 in L japonicus seedlings. Panel A: there is no swelling observed at the base of the hypocotyl of a Lotus seedling that has not been infected with LBA 1334 4 days after sectioning of the main root. In contrast, a slight swelling and root hairs appears in the area of wounding in a seedling 4 days after sectioning of the main root at the base of the hypocotyl and subsequent infection with LBA 1334 (panel B). Panels C, E, and G show GUS expression as blue precipitate using 5-bromo-4chloro-3-indolyl-β-glucoronic acid as substrate in roots transformed with CaMV35S-gus:intron cloned in various binary vectors. GUS histological staining was performed as described in Scarpella et al (2000). Panel C shows that all emerging hairy roots of a Lotus seedling were tranformed with LBA1334 carrying GUS as reporter gene cloned in pCAMBIA1301. Note that GUS is also expressed in loci that have not yet differentiated into hairy roots. Depending on the binary vector used, 5-10% of the transformed plants will show GUS expression in all emerging roots, the rest of the transformed plants wil present a combination of GUS-stained and non-stained roots (see Table 1 for more details). Staining was performed 7 days after transfer of the*

*seedling to HRE medium. Panel E shows blue roots of seedlings transformed with LBA1334 carrying gus in a pPZP derivative and panel D, roots emerging in control seedlings, infected with LBA 1334, that do not show blue staining. GUS histological staining was performed 10 days after transfer to HRE. As many as 20 roots can be initiated, however only a few (2-10 roots) grow rapidly and produce lateral roots at this stage of development. Panel F shows the results of GUS staining of a control plant tranformed with LBA 1334 carrying pBin19 and panel G, the staining of a plant tranformed with LBA 1334 carrying pBin19 CaMV35S-gus:intron and showing GUS activity in nodulated hairy roots. Note that in both cases nodules are concentrated on the most developed root, which in 60-70 % of the cases, is transformed. Predominance of 1 or 2 roots is apparent after 2-3 weeks growth in HRE, clay particles or Jensen-agar plates. Staining of plants in panels F and G was performed 5 weeks after inoculation with M. loti strain R7A and growth of roots in clay particles. Bars in panels A, B, C= 1mm; in panels D, E= 4.5 mm; in panels F, G= 10 mm. See colour plate 4 (A-G) for this figure in colour.*

## *In vitro* culture of GUS-expressing hairy roots and Southern blot analysis

To analyse the number of T-DNA inserts of transformed roots, 4-6 root tips (approximately 0.3 mm) were cut from lateral roots of individually sectioned nodulated hairy roots before staining for GUS activity. Root tips were placed on solidified half strength Murashiga and Skoog (MS) medium containing 3% sucrose, 300 µgmL$^{-1}$ cefotaxime, 100 µgmL$^{-1}$vancomycin and 50 µgmL$^{-1}$ G418, using 94X16 mm plates (CA Greiner & Shöne Ges. m.b. H, Kremsmünster, Austria, article 633102). Root tips were covered with a drop of half strength MS medium containing the mentioned antibiotics, to avoid bacterial overgrowth. After overnight incubation at 20$^{o}$C in the dark, excess of liquid was removed, and root tips were allowed to dry. Root tips corresponding to positive GUS staining roots were grown for 2-3 months in the dark, with transfer to fresh medium every 2 weeks. Bacterial growth was not observed after 4-6 transfers. G418 seems to retard root growth, and in some experiments, it was omitted or substituted by timentin 100 µgmL$^{-1}$.

DNA was isolated from 0.1-0.2 g root tissue as described by van Slogteren et al. (1987). This procedure yielded approximately 2µg DNA, of which 1µg was used for Southern blot analysis. Other DNA extraction procedures we tried yielded DNA of poor quality that either could not be digested by restriction enzymes or resolved properly by electrophoresis.

Southern analysis of 12 independent transformation events obtained with the pPZP111-derivative, some of which derived from the same composite plant, resulted in 75% roots containing one T-DNA integration; 17%, two T-DNA integrations and 8% more than two T-DNA integrations. These results show that pPZP vectors carried by LBA 1334 are excellent tools to deliver the DNA of interest into hairy roots of *L japonicus*.

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