Chapter 2.1

METHODS FOR STUDYING NODULE DEVELOPMENT AND FUNCTION

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Interaction between Lotus japonicus and Mesorhizobium loti results in the development of a specialised organ: the root nodule. Lotus root nodules develop from de-differentiated root cells, which form a meristem that undergoes a limited number of cell divisions. The result is a determinate, roughly spherical organ. Invading rhizobia colonise cells in the nodule cortex, each of which ultimately accommodates many thousands of nitrogen-fixing bacteria called bacteroids. Differentiation of both plant and bacterial cells is crucial for symbiotic nitrogen fixation (SNF), and genetic defects in either partner can compromise SNF. Identification of Lotus and M. loti genes that are necessary for nodule development and function is a major focus of current research. This chapter briefly describes the major steps in Lotus nodule development and differentiation, before presenting methodologies that are used routinely to characterise these processes in wild type and mutant interactions.

LOTUS NODULE DEVELOPMENT AND DIFFERENTIATION

Lotus japonicus is a diploid, perennial legume with a natural habitat in the Far East (Handberg et al., 1992). As most leguminous species, Lotus interacts symbiotically with the beneficial soil bacteria to form root-derived organs, the nitrogen fixing nodules. Fast growing strains of Mesorhizobium loti (e.g. NZP2235, JRL501), the
broad host-range *Rhizobium* strain NGR234, as well as some slow growing *Bradyrhizobium* spp. have been reported to nodulate *Lotus*. However, *M. loti* is the most commonly used species since it is an effective microsymbiont, and the complete nucleotide sequence of the *M. loti* strain JRL501 (MAFf303099) genome has recently become available (Kaneko et al., 2000).

Like soybean, *Lotus* forms spherical root nodule structures of the determinate type (Figure 1). These nodules are characterized by lack of persistent meristematic activity, a developmental feature that distinguishes them from the elongated, indeterminate root nodules of temperate legumes such as pea and alfalfa. However, the organogenesis of *Lotus* nodules has been shown to combine some features of indeterminate and determinate nodule development (van Sprousen et al., 2001).

*Lotus japonicus* responds to *M. loti* by initiating a complex developmental program for nodule organogenesis within the susceptible zone of the root system. The morphogenic signals of bacterial origin, called nodulation (Nod) factors, have been characterized from several different *M. loti* strains (Lopez-Lara et al., 1995; Olsthoorn et al., 1998; Niwa et al., 2001). They were shown to consist of a mixture of lipochitin oligosaccharide (LCO) molecules that are synthesized and secreted from the bacteria in response to an as yet uncharacterized inducer molecule(s), likely to represent isoflavonoids, derived from *Lotus* roots. The major component of the LCO mixture was found to be N-acetylglicosamine pentasaccharide in which the non-reducing residue is N-acylated with a C18:1 acyl moiety, N-methylated, and carries a carbamoyl group, while the reducing end is substituted with 4-O-acetylfucose (Niwa et al., 2001). Ectopic application of the LCO mixture on *Lotus* roots incites various cellular and molecular responses that are reminiscent of the early responses to Nod factors and/or rhizobial infection in other legume species (Lopez-Lara et al., 1995; Niwa et al., 2001).
The earliest responses to Nod factors described in Lotus include membrane depolarization, extra-cellular alkalinization and calcium spiking in root hair cells (Harris et al., 2003; Radutoiu et al., 2003). These physiological reactions occur within a few minutes upon application of purified Nod factors and/or living bacteria to the root system and were shown to depend on the presence of a functional root perception apparatus that involves NFR1/NFR5 LysM receptor-like kinases (Madsen et al., 2003; Radutoiu et al. 2003).

On the cytological level, cells of the epidermis, cortex and root pericycle respond to the presence of *M. loti* and/or application of Nod factors by initiating various growth patterns. In the epidermis, root hair cells deform by tip swelling, branching and curling, leading to the formation of the typical “shepherd’s crook” structures (Szczyglowski et al., 1998, Niwa et al., 2001). These structures entrap the bacteria and serve as a starting point for initiation and growth of the infection thread (IT). Detailed microscopic examination of infected root hairs revealed the presence of bona fide ITs originating at the curled tip and extending the full length of the root hair (Figure 2; Szczyglowski et al., 1998).

![Figure 2. Brightfield micrograph of *L japonicus* nodule 10 days after inoculation with *M. loti* strain NZP2253 carrying a hemA: LacZ reporter gene fusion. Roots were stain for β-galactosidase activity, cleared, and examined using brightfield microscopy. See colour plate 2 (E) for this figure in colour.](image)

The Lotus ITs were shown to be tubular structures consisting of a multilayered fibrillar cell wall, an overlying enclosure membrane continuous with the cytoplasmic membrane of the infected host cell, and a lumen containing vegetative bacteria embedded in an amorphous matrix. Unlike many legumes, Lotus forms very broad infection threads (Figure 3) that extend from infected root hairs through polarized cortical cells of the outer cortex to the meristematic cells of the nodule primordium. Thick and prominent infection threads, usually observed in association with indeterminate-type nodulation, spread between and inside Lotus cortical root cells forming multiple, narrow lateral branches that initiate host cell invasion (Szczyglowski et al., 1998, van Spronsen et al., 2001).
Figure 3. Transmission electron micrographs of a cross-section (A) and longitudinal section (B) of infection threads containing bacteria in *L. japonicus* roots inoculated with *M. loti* strain NZP2235.

In the root cortex, the cells assume an interesting position dependent pattern of dedifferentiation. The outermost cortical cells positioned immediately underneath the infected root hairs either do not divide or make a single anticlinal division. They undergo significant swelling with a concomitant polarization of the cytoplasm in a radial direction to give rise to so-called cytoplasmic bridges (Niwa et al., 2001; von Spronsen, 2001). The formation of cytoplasmic bridges has been observed in the first, second and only occasionally in the third cortical root cell layer (van Spronsen et al., 2001). This type of cell polarization is assumed to guide the progression of the infection thread towards the underlying nodule primordium (Szczyglowski and Amyot, 2003 and references therein).

The third cortical cell layer, as opposed to the outer cortical layer typically involved in the determinate nodule initiation, is proposed to give rise to the first cell divisions leading to the formation of nodule primordia in inoculated Lotus roots (van Spronsen et al., 2001). The fully activated cortical cells of the inner cortex are characterized by enlarged, centrally located nuclei (Figure 4), and contain transvacuolar cytoplasmic strands radiating from the nucleus to the cell wall. These cells undergo successive divisions, which during further development spread to surrounding cell layers, and eventually, give rise to a clearly defined nodule meristem. Incoming ITs ramify within the meristematic region of the nodule primordia where endocytosis of the bacteria is initiated. The release of the vegetative bacteria into the cytosol of the host plant cell has been shown to occur through small erosion zones in the ITs. Individual bacterial cells migrate from within the IT to infection droplets and to the cytosol of the host cells (Figure 5; Szczyglowski et al., 1998). They remain separated from the host cytoplasm by the peribacteroid (symbiosome) membrane. In wild type Lotus nodules, only a subtle morphological transition from the vegetative to bacteroid states occurs, such that the *M. loti* bacteroids undergo a slight increase in size while retaining the same straight rod shape as vegetative cells (Szczyglowski et al., 1998, Suganuma et al., 2003; Figure 6). However, branched and pleomorphic-shaped bacteroids were occasionally observed (Szczyglowski et al., 1998).
Lotus nodules initiate nitrogen fixation approximately 10 days after inoculation with *M. loti*, coinciding with the emergence of young nodules from the root cortex. Twenty one days after inoculation, the mature, spherical, nodules display a characteristic histology consisting of a large central nitrogen fixing area, primarily composed of the bacteroids-containing cells, surrounded by a concentric layer of uninfected nodule cortex. Together with peripherally located vascular elements and the apparent lack of a discernible meristem, these cytological features typify a determinate type of nodule (Szczyglowski et al., 1998).
Although certainly variable and dependent on particular growth conditions used, Lotus establishes a homeostasis of symbiotic root development by limiting the extent of nodulation events, such that only a defined number of nitrogen fixing nodules is formed (Wopereis et al., 2001). Three weeks after inoculation with *M. loti*, 10-15 mature nitrogen fixing nodules can be found on the upper portion of the wild type Lotus root grown under nitrogen limiting conditions (Figure 7). The mechanism involved in this interesting homeostatic regulation is currently being characterized on the molecular level and at least with respect to one aspect of the mechanism involved, a strict requirement for a shoot-derived Har1 receptor kinase function was recently reported (Wopereis et al., 2001; Krussel et al. 2002; Nishimura et al. 2001).

**PLANT GROWTH SYSTEMS FOR NODULATION STUDIES**

A variety of substrates and growth conditions are used to culture inoculated Lotus plants for studies of nodulation and symbiotic nitrogen fixation. Growth substrates include course sand, clay beads, and solidified agarose, some of which are described elsewhere in this book. In this chapter, a glass-slide system that facilitates studies of the initial stages of infection, and a plastic pillow system for the production of mature nodulated plants are described.

**Glass slide culture of seedlings for microscopy**

Liquid culture of seedlings on a slide glass is useful for observation of roots under a microscope without damaging root hairs. This technique can be adopted for root hair deformation assay by application of LCOs (Niwa et al., 2001) or inoculation of *M. loti*, or pharmaceutical assays. This technique can be adopted for root hair deformation assay using LCOs (Niwa et al., 2001) or rhizobium application, or pharmacological assays. This system is useful for studying early infection and

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**Figure 6.** Transmission electron micrographs of *L. japonicus* nodule infected cell containing many symbiosomes (A), and a close up of *M. loti* bacteroids surrounded by peribacteroid membranes (B).
nodulation events, but is not appropriate for later stages when plants become too large.

Figure 7. *L. japonicus* nodulation phenotype 3 weeks after inoculation with *M. loti* strain NZP2235. See cover for this figure in colour.

**Equipment and reagents**
- Glass slides and cover slips
- Silicone caulk
- Staining jars
- Forceps
- B & D medium

**Method**

1. Preparation of Fåhraeus slides
   a. Put roughly 10 µl of silicone caulk at each corner of a cover slip.
   b. Set the cover slip on a glass slide in a way keeping 1 mm space between them, and 1 cm above from the bottom of the glass slide (Figure 8).
Figure 8. Fåhraeus glass slide with cover slip.

c. Let them still for a day in order to solidify silicone.
d. Sterilize by autoclave in a glass jar filled with distilled water.

2. Transfer of seedlings to the slides
   a. Put 50 ml of B & D medium in a sterilized staining jar.
   b. Using forceps, transfer seedlings to the slide one by one, without damaging root hairs (Figure 9). Select only those with straight roots (Miyakojima, 2 day old seedlings; Gifu, 3 day old seedlings as in the section of spot inoculation).

Figure 9. Seedlings in the slide.

c. Insert slides with seedlings to the staining jar (Figure 10).

d. Cover the bottom half of the jar with black paper and aluminium foil to avoid light to roots (Figure 11), culture them at 26°C, 16h light/23°C, 8h dark regime.

Note: We set seedlings four days before applying Nod factors or other chemicals in order to avoid potential wounding responses.
The pillow growth system

The pillow system allows effective nodulation analysis of a large plant population with a relatively small area needed for growing the samples. As several plants can be grown simultaneously, the time-consuming cleaning of root tissue is reduced substantially.

To observe and screen plants for nodule phenotype, it is necessary to germinate and establish the plants on agar media, then transplant them to soil after about a week. Root growth and nodulation kinetics using this method resemble those observed in plants grown in vermiculite/sand mixtures.
I. Sowing seeds in agar medium

Equipment and reagents

- Plastic tray
- 50 ml plastic tube
- Germination medium: 0.8% (w/v) Bacto agar
- Bleach solution: 10% NaClO in sterile distilled water
- Sterile distilled water
- Sterile filter paper
- Sterile Petri dishes or rectangular plastic plates
- Parafilm

Method

1. Prepare the germination medium by autoclaving at 121°C for 15 min and then pour into pre-sterilized Petri dishes.
2. Place the seeds in a 50 ml plastic tube and add 10-15 ml concentrated sulfuric acid. Vortex vigorously for 10-15 min and then pour the solution into a container of tap water. Rinse the seeds at least three times.
3. Put the seeds in a clean plastic tube and add the bleach solution. Shake the container gently for 10 min.
4. Pour out the bleach solution and then wash 4-5 times with sterile water. Keep the seeds in sterile distilled water and shake the container gently for 2-3 hr, then discard the water.
5. Place the seeds in sterile filter paper to dry, and transfer the seeds in germination medium (work in laminar flow hood and use sterile forceps). Seal the Petri plates with Parafilm and incubate in growth chamber under 16-h-light/26°C and 8-h-dark/23°C cycle with 60°C relative humidity.
6. Keep the plates under dark conditions for 2 days and then allow the seeds to germinate for another 7-10 days (i.e. once two to four true leaves have formed).

II. Growing seedlings in the pillow system

Equipment and reagents

- Plastic tray: 30 x 10 x 10 cm
- Pillow bags: polypropylene tea packs (120 x 95 mm) or nylon bags
- Vermiculite:perlite mix (6:1, v/v)
- Broughton and Dilworth (B&D) medium (Table 1)
- 10 µM KNO₃
• Bleach solution: 10% commercial bleach diluted in distilled water
• Saran wrap
• 2 to 3-day-old culture of *Mesorhizobium loti* strain

**Method**

1. Fill the pillow bags with vermiculite:perlite mix and autoclave at 121°C for 20 min.
2. Soak the pillows in sterile B&D nutrient solution containing minimal nitrate concentration (10 µM KNO₃) until the substrate has absorbed the liquid.
3. Sterilize the plastic tray with bleach solution and wash several times with distilled water. Place 10-15 pillow bags side by side in the plastic tray (Figure 12).

![Figure 12](image1.png)

*Figure 12. The pillow system for growing *L japonicus* plants. Pillow bags containing a vermiculite-perlite mixture are placed side by side in the narrow tray.*

4. Sow the seedlings in between individual pillows (five plants per row, Figure 13).

![Figure 13](image2.png)

*Figure 13. *L japonicus* seedlings in between pillows*
5. Incubate the trays in growth chamber. Cover the trays with Saran wrap for a few days to maintain high humidity.

6. After 2 days in the pillow system, inoculate roots with \( M \; loti \) culture at a cell density of \( 10^6 \) cells/ml.

7. Water the plants with B&D nutrient solution (without nitrate) for at least 2 weeks and then with distilled water for the succeeding weeks. Avoid having the growth condition overly wet, or overly dry.

8. For nodule examination, carefully remove the pillows individually taking care not to damage or cut the nodulated root parts.

9. Place the plants in a clean dish with water and examine using stereomicroscopy.

<table>
<thead>
<tr>
<th>Stock solutions</th>
<th>Solution component</th>
<th>2000x stock concentration</th>
<th>Concentration in final solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>CaCl(_2)</td>
<td>2.0 M</td>
<td>1.0 mM</td>
</tr>
<tr>
<td>B</td>
<td>KH(_2)PO(_4)</td>
<td>1.0 M</td>
<td>0.5 mM</td>
</tr>
<tr>
<td>C</td>
<td>Fe-citrate(^b)</td>
<td>0.02 M</td>
<td>10 ( \mu )M</td>
</tr>
<tr>
<td>D</td>
<td>MgSO(_4)</td>
<td>0.5 M</td>
<td>0.25 mM</td>
</tr>
<tr>
<td>E</td>
<td>K(_2)SO(_4)</td>
<td>0.5 M</td>
<td>0.25 mM</td>
</tr>
<tr>
<td>F</td>
<td>MnSO(_4)</td>
<td>2 mM</td>
<td>1.0 ( \mu )M</td>
</tr>
<tr>
<td>G</td>
<td>H(_3)BO(_3)</td>
<td>4 mM</td>
<td>2.0 ( \mu )M</td>
</tr>
<tr>
<td>H</td>
<td>ZnSO(_4)</td>
<td>1 mM</td>
<td>0.5 ( \mu )M</td>
</tr>
<tr>
<td>I</td>
<td>CuSO(_4)</td>
<td>0.4 mM</td>
<td>0.2 ( \mu )M</td>
</tr>
<tr>
<td>J</td>
<td>CoSO(_4)</td>
<td>0.2 mM</td>
<td>0.1 ( \mu )M</td>
</tr>
<tr>
<td>K</td>
<td>Na(_2)MoO(_4)</td>
<td>0.2 mM</td>
<td>0.1 ( \mu )M</td>
</tr>
</tbody>
</table>

Table 1. Broughton and Dilworth (1971) nutrient solution. *Adjust the pH to 6.8 with KOH.  
\(^b\)Dissolve Fe-citrate with heating and keep in dark bottle

**MICROSCOPY METHODS**

**Light-microscope analysis of bacterial infection and colonization of root cells using lacZ-expressing rhizobia and X-Gal staining**

Investigation of infection process is important for analysing physiologic and genetic aspects of nodulation. Ethylene and nitrogen compounds like nitrate are known to inhibit nodulation, and there are several mutants both in plants and rhizobia that show defect in infection. The method shown here is conventional but powerful tool to see colonization of rhizobium in shepherd crook and infection thread development.
Equipment and Reagents

- 15 ml or 50 ml plastic tubes
- Incubator at 30ºC
- 1XPBS, pH7.0
- 2% Glutaraldehyde in 1XPBS
- 20 mg/ml X-Gal in N-Dimethylformamide (keep at -20ºC)
- 50 mM Potassium hexacyanoferrate (III) (4ºC)
- 50 mM Potassium hexacyanoferrate (II) trihydrate (4ºC)

Method

1. Prepare inoculated plants with *Mesorhizobium loti* harbouring constitutively expressing lacZ (Tansengco et al., 2003). Infection threads can be observed after three days of inoculation, and nodules are visible after seven days. If plants are grown in vermiculite/perlite mixture, remove them carefully in PBS by forceps. Vermiculite is easy to remove after soaking plants in PBS for several minutes. Similarly, if plants are grown on agar plate, remove agar.

2. Add glutaraldehyde solution to a plastic tube, put samples in, and then vacuum for 20-30 minutes. Subsequently, let samples keep one hour at room temperature.

3. Wash samples with PBS for three times.

4. Prepare staining solution (0.8 mg/ml of X-Gal, 2.5 mM of potassium ferricyanide in 0.2x PBS) and add the solution to the samples.

5. Vacuum 3-5 minutes (longer vacuum is necessary for staining inside the nodule). Normally, when the samples are wilted, vacuum is enough.

6. Keep samples in staining solution for overnight at room temperature.

7. Transfer samples to PBS.

8. Root tissue can be cleared by chloral hydrate solution mentioned below.

9. Observe samples under stereomicroscope or inverted light microscope.

*Note:* For observation of infection threads, a shorter period (90 min to 3 hrs) of staining is better, because endogenous activity of lacZ in root stele interferes with the observation of stained infection threads.

Clearing tissues with chloral hydrate for light microscopy

Tissue clearing is useful for the analysis of internal structures of whole mount plant tissue. Presented here is a simple clearing technique that can then be observed under DIC (differential interference contrast) microscopy. This is an efficient way to examine root and nodule development, as well as other non-ovule materials, without
sectioning. The protocol can also be used for clearing LacZ- or GUS-stained materials, after chlorophyll is removed by 70% ethanol.

**Equipment and reagents**

- Clearing solution: chloral hydrate:glycerol:water, 8:1:2 (dissolve by stirring for 1 hr)
- Fixative: ethanol:acetic acid, 9:1 (v/v)
- Sterile distilled water
- Ethanol/water solution: 90%, 70%, 50%, 30% (v/v)
- Glass vials
- Light microscope

**Method**

1. Place the tissues in a glass vial and immerse in ethanol:acetic acid solution.
2. Fix the samples under vacuum for 10-15 min and then overnight at room temperature.
3. Discard the fixing solution and rinse briefly with sterile water.
4. Immerse the tissues in ethanol/water series in the following steps: 90%, 70%, 50%, and 30% (v/v) for 30 min each.
5. Rinse the samples briefly with sterile water and place in clearing solution for several hours or overnight depending on the specimen. Tissues can be stored in clearing solution at room temperature for several weeks.
6. Observe the samples under a light microscope (Figure 14).

![Figure 14](image)

**Figure 14.** Cleared tissues showing successive stages of nodule development. A: Cortical cell division, B: Bump, C: Developing nodule. See colour plate 2 (A, B, C) for this figure in colour.
Glycol methacrylate (Technovit) embedding of plant tissues for fluorescence microscopy

Thin sectioning using glycol methacrylate (GMA)-based embedding medium (commercially available as Technovit 7100) is especially suited for the analysis of many biological samples. The polymerization of GMA is initiated by means of a barbituric acid derivative in combination with chloride-ions and benzoyl peroxide. Its low viscosity for complete and rapid infiltration and the hardness of the resulting polymer allow thinner sections to be cut providing clear images of cellular detail. The protocol is simple giving a minimum processing time of only a few days. Moreover, staining and enzymatic reactions using standard methods are possible without removing the resin.

I. Fixation and embedding of plant tissues

Equipment and reagents

- Glass vials
- Vacuum dessicator
- Rotator (4 rpm)
- Sample embedding molds
- Fixative: 4% (w/v) paraformaldehyde in 20 mM sodium cacodylate buffer pH 7
- Ethanol: 30%, 50%, 70%, 80%, 90%, 95%, 99%, 100% (v/v)
- Technovit 7100 embedding kit (Heraeus Kulzer, Wehrheim, Germany)
- Technovit 3020 kit (Heraeus Kulzer)

Method

1. Preparation of Fåhraeus slides
   a. Put roughly 10 µl of silicone caulk at each corner of a cover slip.
   b. Set the cover slip on a glass slide in a way keeping 1 mm space between them, and 1 cm above from the bottom of the glass slide (Figure 8).
   c. Let them still for a day in order to solidify silicone.
   d. Sterilize by autoclave in a glass jar filled with distilled water.

2. Fixation
a. Cut small pieces of tissue (<5 mm). Try to keep the sample size small to facilitate processing, i.e. fixation, infiltration, and sectioning.

b. Prepare fresh 4% (w/v) paraformaldehyde solution by dissolving the powder to the buffer with heating in fume hood and add 1-2 drops of 1N NaOH solution. Adjust pH after cooling down. Fix samples for 30 min under vacuum.

c. Change to fresh fixative and fix overnight at 4°C.

3. Dehydration
   a. Dehydrate tissue in an ethanol/water series at RT in the following steps: twice in 30%, 50%, 70%, 80% (v/v) for 15 min each, twice in 90%, 95%, 99% for 5 min each, then in 100% for 1 hr.
   b. Use a rotator (4 rpm) or a rocking platform for agitation.

4. Infiltration
   a. Prepare infiltration solution consisting of 100 ml Technovit resin (2-hydroxyethyl methacrylate) and 1 g Hardener I (benzoyl peroxide, as accelerator).
   b. Mix until the powder is completely dissolved and store solution in dark bottle. At 4°C, the solution remains stable for approximately 4 weeks (avoid air).
   c. Immerse the tissue in fresh 1 ml absolute ethanol and add 250 µl infiltration solution every 15 min.
   d. Mix the sample after adding the infiltration solution and keep the tissue vials in rotator.
   e. Remove half volume of the solution every hour (for a total of 3 hr) making the solution 50% infiltration solution after 1 hr, 75% after 2 hr, and 82.5% after 3 hr.
   f. Leave the sample in 100% infiltration solution overnight in a rotator.
   g. Change to fresh infiltration solution and leave for 2-3 hr with agitation.

5. Embedding
   a. Prepare embedding solution consisting of 15 parts of infiltration solution and 1 part of Hardener II.
   b. Mix the solution and immediately pour in a suitable mold (we use a plastic film case) and add the samples. Arrange the specimen appropriately and allow polymerizing for at least several hours.

6. Mounting
   a. Mix Technovit 3040 in a volume ratio of 2 parts powder to 1 part liquid.
b. Mix vigorously and immediately pour on top of the embedded sample.

c. Remove the block from the mold after about 10 min. If the sample remains soft due to high temperature and humidity, store the samples in auto-dry desiccators for one to several days.

II. Sectioning and staining

Equipment and reagents

- Fret saw
- Epoxy glue
- Plastic or metal stubs
- Razorblade
- Ultramicrotome
- Sterile distilled water
- Glass slides and cover slips
- Forceps
- Hotplate
- 0.05% Toluidine Blue O in 1x PBS (137 mM NaCl, 8.10 mM Na₂HPO₄·12H₂O, 2.68 mM KCl, 1.47 mM KH₂PO₄, pH 7.4)
- DAPI (4’,6-diamidino-2-phenylindole) staining solution: 10 µg/ml in vectashield antifade mounting medium (Vector Laboratories, Inc.) with 5 µg/ml fluorescent brightener 28 (Sigma-Aldrich)

Method

1. Separate embedded tissues in plastic blocks by sawing the block into small pieces using a fret saw.
2. Mount the blocks onto metal or plastic stubs using epoxy glue.
3. Trim the edges of the block to a rectangular face using a razor blade.
4. Prepare triangular glass knives using a knife maker machine.
5. Set the block and glass knife in the ultramicrotome such that the cutting face of the block is parallel with the knife-edge.
6. Cut sections at 1-2 µm thick and float individual sections one at a time on distilled water on a cover slip by forceps.
7. Handle each section with a forceps and let go sections just before it touches the water.
8. Place cover slips at 50°C on a hot plate and let the water evaporate.
9. Stain with toluidine blue solution and observe under brightfield light microscopy (Figure 15).
Figure 15. Longitudinal section of *L.* japonicus anther showing pollen mother cells. TBO staining.

10. Alternatively, for fluorescent microscopy, stain with DAPI solution containing fluorescent brightener to stain the nuclei and cell wall, respectively (Figure 16)

Figure 16. *Lotus japonicus* nodule 2 weeks after infection with *Mesorhizobium loti*. DAPI staining. See colour plate 2 (D) for this figure in colour.
Spot inoculation

To investigate the initial events of nodule development, spot inoculation is a powerful method in terms of temporal observation because it is easy to identify the focus of nodule initiation. In contrast to the slide inoculation method described below, it is not easy to evaluate the quantity of root hair deformation using this method, but the slide inoculation hardly induces nodules and they are not spatially fixed. For inducing nodule primordia, one can apply rhizobium (Imaizumi-Anraku et al., 2000) as well as LCOs, but the latter fail to induce complete (empty) nodules (Niwa et al., 2001). In general, MG-20 Miyakojima forms bigger nodule primordia than B-129 Gifu accession by LCOs spot. For inoculation, we use agarose for matrix to spot rhizobium or LCOs, other application method using quartz sands (van Spronsen et al., 2001) however would be also possible.

Equipment and reagents

1. Square Petri dish (either 8 cm x 12 cm or 8 cm square)
2. Low melting agarose (SeaPlaque GTG agarose, BMA)
3. TY medium
4. B & D medium
5. LCOs mixture from M. loti JRL501 harbouring pMP2112 (Niwa et al., 2001)

Method

1. Preparation of seedlings for spot inoculation
   a. Treat seeds with sulfuric acid (conc) for 5 to 8 min (depending on the seed condition) followed by extensive washing by tap water. Scarification by a piece of sandpaper is the alternative.
   b. Sterilize seeds with sodium hypochlorite solution (1% active chlorine) for less than 10 min, and rinse by sterilized water for three times.
   c. Imbibe seeds in sterile water for a few hours at room temperature, with gentle shaking.
   d. Transfer seeds to Petri dish containing 0.8% Bacto Agar (BD). Cover the dish with aluminium foil. Incubate at 26°C-16h-light/23°C-8h-dark regime. Remove foil after one day (Miyakojima) or two days (Gifu), and incubate one extra day.
   e. Transfer seedlings to the square Petri dish containing B&D medium solidified with 1% Bacto Agar.
   f. Cover the dish with black paper and aluminium foil (leave top 1 cm open), put the dish vertically, and incubate for 2 days (Figure 17). Addition of AVG is of choice, use 10 µM in case.
2. Preparation of spot solution, Rhizobium
   a. Inoculate *M. loti* from plate to 3 ml of TY liquid medium, culture at 28ºC for 2 days.
   b. Spin down culture by centrifuge at 8000 rpm, 2 min, 4ºC.
   c. Wash pellet with saline (0.8% NaCl) 2 times, adjust the concentration to O.D.\(_{600}\)=0.1.
   d. Mix 25µl of the suspension with 50 µl of sterilized low melting agarose (1%).

3. Preparation of spot solution, LCOs:
   a. Mix 25µl of LCOs (10\(^{-5}\) to 10\(^{-3}\)M) with 50 µl of sterilized low melting agarose (1%).

4. Spot inoculation
   a. Pipette out 10µl of the solution above, and spot 1 µl of it to the root tip, using micropipette.
   b. Wrap the rid of the dish with Parafilm. Use surgical tape (Micropore: 3M) at the top to avoid excess humidity.
   c. Set black paper and aluminium foil again as above, and incubate.
   d. After one week, developing nodule can be observed by naked eye (rhizobium inoculation). In case of LCOs, 10 days are enough to recognize the nodule under stereomicroscope.

*Notes:* Higher concentrations of LCOs bring better nodulation, but not 100%. Normally more than 50% of the spotted seedlings show nodulation. Nodules can be observed 0 to 2 cm below the spot point. In the original papers, the authors...
recommended adding black ink so that the spot point can be easily recognized. In our experience, however, black ink inhibits root elongation, so we do not add it. Holding the dish up to the light can identify the spot point.

**ACETYLENE REDUCTION ASSAY FOR NITROGENASE ACTIVITY**

Biological nitrogen fixation is assessed using several methods, such as Kjeldahl analysis, $^{15}$N-incorporation assays, acetylene reduction assays, and $\text{H}_2$-evolution assays. Of these methods, the closed acetylene reduction assay is simple, rapid, and sensitive (Hardy et al. 1968; Vessey 1994), and has been widely used for estimating the nitrogen-fixing activity of legumes. This assay takes advantage of nitrogenase’s high affinity for acetylene, and in the presence of acetylene total electron flow through nitrogenase is virtually used to reduce acetylene to ethylene. The ethylene produced can be detected by hydrogen flame ionization after gas chromatography (Figure 18).

**Reagents and apparatuses**

- Acetylene
- Ethylene (for standard)
- 35-mL vial (Any vials with different volumes are available if the vial can be sealed with a cap, from which a gas sample can be withdrawn with a syringe.)
- Serum cap
- 1-mL disposable syringe with a needle (25G x 1’’)
- Glass column (2 mm i.d. x 2 m) packed with Porapak N (Waters, Milford, MA, USA)
- Gas chromatograph equipped with a hydrogen flame-ionization detector (FID)
Integrator (for determination of the peak area separated by gas chromatography).

**Procedures**

- Detach a nodulated root from a freshly harvested plant.
- Place the nodulated root in a 35-mL vial and close with a serum cap. A single nodulated root is enough for determination of activity using the 35-mL vial.
- Withdraw 3.5 mL of air from the closed vial and inject the same volume of acetylene gas to be 0.1 atmosphere of acetylene in the vial.
- Immediately withdraw 0.5 mL of gas sample from the closed vial and measure the concentration of ethylene contaminated in the 0.1 atmosphere of acetylene injected in the vial with gas chromatograph.
- Incubate the closed vial containing the nodulated root in darkness at 25°C.
- After 30 min, withdraw 0.5 mL of gas sample and measure the concentration of ethylene.
- Based on standard ethylene, calculate the ethylene produced during the incubation after subtracting the ethylene contaminated in acetylene gas.

**Notes**

- Acetylene cylinder contains a small amount of ethylene and the ethylene impurity should be determined daily since it varies inversely with the pressure in the cylinder.
- Preparations of tissue seriously affect acetylene reduction activity (Vessey 1994). The least disturbed, most intact tissue should be used in acetylene reduction assay.
- Rates of acetylene reduction are linear up to 60 minutes, but it is recommended that the assay time should be kept to within 30 minutes (Hardy et al. 1968).
- Nodulated roots evolve endogenous ethylene (Suganuma et al. 1995a), though the amounts of evolution are thought to be negligible compared with acetylene reduction activity determined within 30 min.
- Replacement of air (N₂) with Ar:O₂ slightly increases acetylene reduction activity (Hardy et al. 1968). If required, air in each vial should be replaced by introducing a mixture of Ar:O₂ (0.8:0.2 atmosphere) gas before adding acetylene gas.
MOLecular Markers and Measuring Methods

Nodule development and differentiation are orchestrated by changes in gene expression that occur in a precise temporal sequence. Nodule-specific plant genes that are induced within hours or days after contact with rhizobia are called early nodulin genes, while those that are induced a week or so later, just prior to or following the start of nitrogen fixation are called late nodulin genes. Early nodulins are believed to play important roles during bacterial colonisation or nodule development, while late nodulins presumably fulfil structural or functional roles during differentiation and/or nitrogen fixation. Sequential changes in rhizobial gene expression also accompany nodule development and differentiation. Because of their well-defined temporal expression patterns, many plant and bacterial nodule-induced genes, or the proteins that they encode, have been used as molecular markers for different stages of nodule development. Such markers are especially useful for studies with plant or bacterial mutants that are defective in nodulation or nitrogen fixation, as they help to identify the stage at which nodule development or differentiation is impaired. Expression of marker genes in wild type and ineffective nodules has been examined by Northern analysis (Purdom and Trese 1995; Kato et al. 2002), in situ hybridization (Banba et al. 2001; Kawashima et al. 2001), immunoblotting (Egli et al. 1991; Romanov et al. 1995; Suganuma et al. 2003) and use of bacterial reporter gene fusions (Voroshilova et al. 2001). The following two sub-sections describe the methods of real-time RT-PCR and immunoblotting for measuring gene transcript and protein levels, respectively.

Transcript Profiling Using Real-time RT-PCR

Real-time reverse transcription-polymerase chain reaction (RT-PCR) is an extremely sensitive and quantitative method of measuring gene transcript levels that can be multiplexed to handle transcripts of many different genes in parallel. However, care must be taken to remove all genomic DNA from isolated RNA before RT-PCR measurements (Czechowski et al., 2004).

Materials and Methods

Harvested plant material is typically frozen immediately in liquid nitrogen and stored at -70 °C, prior to use. Total RNA is extracted from frozen organs of Lotus plants using an RNeasy kit (Qiagen, Hilden, Germany). Before cDNA synthesis, 1-2 μg of total RNA is treated with RNase-free DNaseI (Sigma, St Louis, MI, USA), to destroy genomic DNA. DNaseI-treated RNA is subsequently phenol-chloroform extracted, precipitated, and resuspended in 10 μL water. PCR, using gene-specific primers, is performed on the resulting RNA to confirm the absence of DNA. Reverse transcription is then performed with SuperScript™ III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) using an oligo(dT) primer. The efficiency of cDNA synthesis is assessed by real-time PCR amplification of ubiquitin (see Table 2), and only those reactions that exhibit similar efficiencies (similar C_T values for ubiquitin) are analysed further.
PCR reactions can be performed in a 96-well plate with an Applied Biosystems ABI Prism 5700 Sequence Detection System (Foster City, CA, USA), using SYBR Green to monitor dsDNA synthesis. A standard PCR reaction contains approximately 4 ng cDNA template, 10 µL of SYBR Green Mix (Applied Biosystems, Foster City CA, USA), 5 pmol forward and reverse primers, and water to a final volume of 20 µL. The following standard thermal profile is used for all PCR reactions: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 sec and 60°C for 1 min. Data is analysed using SDS 2.0 software (Applied Biosystems, Foster city, CA, USA). For quality control, PCR products are run on a 3 % agarose gel to confirm the presence of a single amplicon. Gene-specific primers can be designed with PrimerExpress software (Applied Biosystems, Foster city, CA, USA), and typically have melting temperatures of 60 +/- 2 and GC content of between 45-55%. Primers are designed to produce short amplicons, typically between 60-150 bp to ensure high PCR efficiencies.

Threshold cycle (C_T) values for each gene transcript are normalized to those of polyubiquitin (GenBank AW720576; see Table 2) before making comparisons between samples (Colebatch et al. 2002). Gene expression ratios are calculated using the following equations:

\[ nCT = C_T - C_{T_{Ub}} \]
\[ \Delta CT = nC_{T_x} - nC_{T_y} \]
\[ nRatio = 2^{-\Delta CT} \]

Where \( C_T \) is the PCR cycle number at which a set threshold value (usually 0.1) of SYBR Green fluorescence is reached, \( nC_T \) is the normalised \( C_T \) value, \( \Delta C_T \) is the difference in normalised \( C_T \) values in sample \( x \) and \( y \), and \( nRatio \) is the ratio of gene transcript level in sample \( x \) compared to \( y \). The last equation assumes a PCR efficiency (E) of 100%. Generally, this is not the case, and several methods are available to estimate PCR efficiency. The first method of measuring PCR efficiency uses template dilutions and the equation \((1+E)=10^{-\frac{1}{slope}}\), as described previously (Pfaffl et al., 2001). The second method uses data obtained from the exponential phase of each individual amplification plot and the equation \((1+E)=10^{slope}\) (Ramakers et al., 2003). If \( E \) is known, the gene expression ratio can be calculated using the equation:

\[ nRatio = (1+E)^{-\Delta CT} \]
Gene Primer sequences (F, forward; R, reverse) Comment
LjUbi F: 5’- TTCACCTTGTGCTCGTCTTC-3’  
R: 5’- AACAACACACACACAGACATCC-3’  Constitutive
LjSbtS F: 5’- ATGAAGACTGAGAAGAATCCC-3’  
R: 5’-GTAATTATATATTTGTCCTCCCACGGAAG-3’  Induced 2-5 hpi
LjNin F: 5’- AATGCTCTTGATCAGGCTG-3’  
R: 5’- AGGAGCCTAAGTGAGTGCTA-3’  Induced 5 hpi
Leghaemo-  
globin F: 5’-AAAGACATGCTCTTTCTTC-3’  
R: 5’- CATTGCTCCTCTTATGCA-3’  Induced 7 dpi
LjEnod2 F: 5’- CAGGAAAACCCACACAGTG-3’  
R: 5’- ATGGAGGCGAATACACTGGTG-3’  Induced 12 dpi
LjKUP F: 5’- CGTTGATTCTGCTCACAGGCAC-3’  
R: 5’- TAAAGCAGCAGATGTCACGAC-3’  Induced 14 dpi

Table 2: Marker genes and corresponding primers for real-time RT-PCR. The polyubiquitin gene, LjUbi is a constitutively expressed gene used for normalization of data (Colebatch et al., 2002). Other genes are used as molecular markers for early or late stages of nodule development and differentiation. The symbiosis-induced subtilase gene, LjSbtS (Martin Parniske, personal communication), and the nodule inception gene, LjNin (Schauser et al. 1999), are induced within a few hours post-inoculation (hpi). The other genes, including LjKUP (Desbrosses et al., 2004), are induced several days post-inoculation (dpi).

**Measuring changes in protein abundance using immunoblotting**

When the appropriate antibody is available, expression of marker genes can be easily and sensitively analysed using immunoblotting. In immunoblotting, methods for detection of target proteins are variable. As an example, visualizations of immunoreactive leghaemoglobin, a representative late nodulin, and nitrogenase in bacteroids with a ProtoBlot immunoblotting system (Promega, Madison, WI, USA) are described (Figure 19).

**Reagents and Apparatuses**

- Grinding medium (50mM Tris-HCl pH7.4, 0.3M sucrose, 1mM EDTA, 5mM DDT, 1mM PMSF)
- Polyclar AT
- PVDF membrane (Immobilon-P membrane; Millipore, Bedford, MA, USA)
- TBS (20mM Tris-HCl pH 7.5, 150mM NaCl)
- TBST (20mM Tris-HCl pH 7.5, 150mM NaCl, 0.05% Tween 20)
- Blocking solution (1% (w/v) BSA in TBST)
• Alkaline phosphate buffer (100mM Tris-HCl pH 9.5, 100mM NaCl, 5mM MgCl₂)
• Colour development solution (BCIP/NBT in alkaline phosphate buffer)
• Primary antibody
• Anti-IgG secondary antibody conjugated to alkaline phosphatase
• Sonicator
• Electrophoresis system
• Blotting apparatus

![Figure 19. Examples of immunoblotting analysis of nitrogenase component I (C-I) (A) and component II (C-II) in a crude bacteroids, and of leghaemoglobin (B) in a host plant fraction isolated from nodules on wild type Gifu (G) and Lotus japonicus sen1 (Ljsym75) mutant (S) plants. For reference, free-living Mesorhizobium loti (M) and root extracts from Gifu plants (R) were also tested, respectively.]

Procedures

Preparation of extracts for crude bacteroids and for plant cytosol

• Homogenize nodules with a mortar and pestle in grinding medium with Polyclar AT powder in a ratio of 0.3 g per g fresh weight of nodules.
• Squeeze the homogenate through four layers of gauze and centrifuge the filtrate at 200 x g for 10 min at 4°C.
• Centrifuge the supernatant at 5,000 x g for 10 min at 4°C and wash the pellet with grinding medium three times.
• Sonicate the final suspension ten times for 10 sec each time at 100W and centrifuge at 16,000 x g for 30 min at 4°C. The resulting supernatant is used as the bacteroid fraction in immunoblotting.
Centrifuge the supernatant, obtained by the centrifugation at 5,000 x g for 10 min, at 16,000 x g for 30 min at 4°C. The resulting supernatant is used as the plant cytosol fraction in immunoblotting.

**Electrophoresis and transfer to membrane**

- Separate the polypeptides by SDS-PAGE (polyacrylamide gel electrophoresis) on a 12% (w/v) polyacrylamide gel. Bacteroid fraction is used for detection of nitrogenase and plant cytosol fraction is for detection of leghaemoglobin, respectively.
- Transfer the polypeptides separated by SDS-PAGE to PVDF membranes electrophoretically (Towbin et al. 1979).

**Immunodetection**

- Incubate the membrane in blocking solution for 60 min to block nonspecific protein binding.
- Incubate the membrane in TBST containing the appropriate dilution of primary antibody for 30 min with gentle agitation. Antibodies against nitrogenase components I and II isolated from *Rhizobium leguminosarum* bv viciae bacteroids (Bisseling et al 1980) were diluted to 1:10,000 and that against leghaemoglobin isolated from soybean nodules (Suganuma et al. 1995b) was diluted to 1:10,000.
- Wash the membrane three times in TBST for 10 min each.
- Incubate the membrane in TBST containing the appropriate dilution of anti-IgG alkaline phosphatase conjugate for 30 min with gentle agitation.
- Wash the membrane three times in TBST for 10 min each wash.
- Rinse the membrane briefly in TBS two times.
- Incubate the membrane in colour development solution.
- Stop the reaction by washing the membrane in deionized water for 15 min when the colour has developed to the desired intensity.

**Notes**

- PVDF membrane, a hydrophobic membrane, must be pre-wet first in 100% methanol or ethanol. After pre-wetting, submerge the membrane to blotting solution until use.
- Do not dry out the membrane during any steps of the procedures after pre-wetting.
- You can store the membrane covered with a plastic sheet at 4°C after blotting. For the dried PVDF membrane, first re-wet in methanol or ethanol followed by floating in TBS.
Detailed procedures for electrophoresis and electrophoretic transfer are usually included with commercial devices.

REFERENCES


