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## Chapter 3.1

### CONCURRENT VISUALIZATION OF *GUS*A AND *LAC*Z REPORTER GENE EXPRESSION

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*Here we describe conditions under which two reporter genes, encoding  $\beta$ -glucuronidase (*gusA*) and  $\beta$ -galactosidase (*lacZ*), can be used to simultaneously distinguish between the sites of expression of plant and bacterial genes, respectively, in symbiotic tissues. These conditions involve the fixation of cytoplasmic proteins with cold methanol before detecting *gusA* expression, and the addition of detergents to the incubation solution in order to inactivate endogenous plant  $\beta$ -galactosidases.*

#### INTRODUCTION

The nodulation of the roots of leguminous plants by rhizobia is the result of concerted gene expression of both symbiotic partners. In plant cells, the timing and localization of genes involved in this process is usually visualized with promoter-*gusA* (Jefferson et al, 1987) fusions. Concurrent with plant gene expression, subsequent staining of rhizobia carrying constitutively expressed *lacZ*-encoding  $\beta$ -galactosidase ( *$\beta$ -gal*) as a reporter gene with a contrasting substrate has been used to localize rhizobia at different nodulation steps (Pichon et al, 1994a).

Here we describe the procedures we follow to obtain double staining of reporter gene activities during nodulation of *Lotus japonicus* roots. We adapted the protocol applied by Pichon et al. (1994a) on nodulated *Medicago truncatula* roots because *Lotus* roots seem to present a barrier for efficient inactivation of endogenous  $\beta$ -galactosidases and rapid penetration of enzyme substrates. We carried out precipitation of cytoplasmic proteins in cold methanol before the detection of plant promoter *gusA* fusions (Wijers et al, 2001). In addition, we added detergents to the

solutions used for the inactivation of endogenous  $\beta$ -galactosidases and for the detection of activities of reporter genes. These modifications gave a reduction of background staining originated by endogenous root  $\beta$ -galactosidases and a shortening of the reaction time of the promoter-reporter gene fusions.

To set up this protocol, we obtained hairy roots of *L. japonicus* by transformation with *Agrobacterium rhizogenes* LBA 1334 containing pPB12B, a derivative of the binary vector pLP100 carrying the *Medicago sativa* *ENOD12B* promoter fused to *gusA*. This construct presented a nodulation-independent expression in lateral roots, as well as induced expression upon inoculation with *Sinorhizium meliloti* in transgenic *M truncatula* plants (Trinh et al, 1998; see Chapter 6.2 for a protocol for hairy roots induction). We took advantage of the expression of *MsENOD12B-gusA* during nodulation of Lotus roots by *Mesorhizobium loti* R7A containing pXLGD4, that carries the constitutively expressed *hemaA* promoter fused to *lacZ* (Leong et al, 1985), for the improvement of concurrent visualization of plant and bacterial reporter genes. A detailed analysis of the expression of *MsENOD12B-gusA* is beyond the scope of this work.

## MATERIALS

### Stock solutions

Stock	Volume prepared	Procedure	Storage
90% (v/v) Methanol	100 mL	Mix 90 mL methanol with 10 mL water <sup>1</sup>	-20°C
0.2 M Na <sub>2</sub> HPO <sub>4</sub> -NaH <sub>2</sub> PO <sub>4</sub> pH 7	1000 mL	Dissolve 22.2 g Na <sub>2</sub> HPO <sub>4</sub> ·2H <sub>2</sub> O and 10.4 g NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O in water, check and correct pH.	100 mL aliquots, sterilized solution at RT
0.2 M Na <sub>2</sub> HPO <sub>4</sub> -NaH <sub>2</sub> PO <sub>4</sub> pH 7.4	1000 mL	Dissolve 29.2 g Na <sub>2</sub> HPO <sub>4</sub> ·2H <sub>2</sub> O and 4.9 NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O in water, check and correct pH	100 mL aliquots, sterilized solution at RT
1% (v/v) Triton X-100	100 mL	Add 1 mL detergent to 99 mL water	25 mL aliquots at -20°C, and liquid at 4°C
1% (w/v) N-laurylsarcosine	100 mL	Dissolve 1 g in water	25 mL aliquots at -20°C, and liquid at 4°C
0.5 M NaEDTA, pH 8	200 ml		
0.2 M K <sub>2</sub> HPO <sub>4</sub> -KH <sub>2</sub> PO <sub>4</sub> , pH 7	1000 mL	Dissolve 21.6 g K <sub>2</sub> HPO <sub>4</sub> and 10.34 g KH <sub>2</sub> PO <sub>4</sub> ·2H <sub>2</sub> O in water, check and correct pH	100 mL aliquots, sterilized solution at RT

Stock	Volume prepared	Procedure	Storage
0.1 M MgSO <sub>4</sub>	100 mL	Dissolve 2.465 g MgSO <sub>4</sub> ·7 H <sub>2</sub> O in water	25 mL aliquots, sterilized solution at RT
1 M KCl	100 mL	Dissolve 7.46 g KCl in water	25 mL aliquots, sterilized solution at RT
25 mg mL <sup>-1</sup> X-gluc (5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid, cyclohexylammonium salt)	5 mL	Dissolve 125 mg X-gluc in 5 mL dimethyl-formamide (DMFO) in a dark glass vial	Keeps for 4-6 months at -20°C
40 mg mL <sup>-1</sup> Magenta-gal (M-gal, 5-Bromo-6-Chloro-3-indolyl-β-D-galactopyranoside) <sup>3</sup> .	2.5 mL	Dissolve 100 mg M-gal in 2.5 mL dimethyl-formamide (DMFO) in a dark glass vial	Keeps for 4-6 months at -20°C

Deionized, sterilized water is used to prepare all solutions. To avoid bacterial contamination, solutions are sterilized for 20 min at 120°C then stored at room temperature (RT). Open bottles are stored at 4°C. M-gal can be ordered from AppliChem [www.applichem.de](http://www.applichem.de) or from [www.biotium.com](http://www.biotium.com).

## Solutions for detecting plant promoter-*gusA* fusions

### GUS fixative

- 90% methanol

### Pi-Ferri buffer

0.1 M Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub> pH 7 containing 1 mM K<sub>3</sub>Fe(CN)<sub>6</sub>. Use within 2 weeks; store in the dark at 4°C.

To prepare	200 mL
0.2 M Na <sub>2</sub> HPO <sub>4</sub> - NaH <sub>2</sub> PO <sub>4</sub> pH 7	100 mL
0.1 M K <sub>3</sub> Fe(CN) <sub>6</sub>	2 mL
water	98 mL

### GUS reaction buffer

0.1 M Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub> pH 7 containing 1 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 1 mM K<sub>4</sub>Fe(CN)<sub>6</sub>, 10mM EDTA, 0.1% Triton X-100, 0.1% N-laurylsarcosine and 0.5 mg mL<sup>-1</sup> X-gluc.

To prepare, just before use	10 mL	50 mL
0.2 M Na <sub>2</sub> HPO <sub>4</sub> -NaH <sub>2</sub> PO <sub>4</sub> pH7	5 mL	25 mL

0.1 M $K_3Fe(CN)_6$	0.1 mL	0.5 mL
0.1 M $K_4Fe(CN)_6$	0.1 mL	0.5 mL
0.5 M NaEDTA	0.1 mL	0.5 mL
1% Triton X-100	1 mL	5 mL
1% N-laurylsarcosine	1 mL	5 mL
25 mg mL <sup>-1</sup> X-gluc	0.2 mL	1 mL
Water	2.5 mL	12.5 mL

### Washing buffer

0.1 M  $Na_2HPO_4$ - $NaH_2PO_4$  pH 7.4

<b>To prepare</b>	<b>200 mL</b>
0.2 M $Na_2HPO_4$ - $NaH_2PO_4$ pH7.4	100 mL
Water	100 mL

### Solutions for the detection of bacterial promoter-*lacZ* fusions

#### Endogenous $\beta$ -gal inactivation buffer

0.1 M  $Na_2HPO_4$ - $NaH_2PO_4$  pH 7.4 containing 1.25% glutaraldehyde. (0.1% Triton X-100, 0.1% N-laurylsarcosine can be added when nodulated roots are being assayed 4 weeks after inoculation). Use electron microscopy grade glutaraldehyde, store bottle at 4°C in the dark. Take volume needed with a needle and syringe.

<b>To prepare, just before use</b>	<b>10 mL</b>	<b>50 mL</b>
0.2 M $Na_2HPO_4$ - $NaH_2PO_4$ pH7.4	5 mL	25 mL
Glutaraldehyde 25%	0.5 mL	2.5 mL
Water	4.5 mL	22.5 mL

#### Z buffer

0.1 M  $K_2HPO_4$ - $KH_2PO_4$  pH 7 containing 1 mM  $MgSO_4$ , 10mM KCl, 0.1% Triton X-100 and 0.1% N-laurylsarcosine.

<b>To prepare</b>	<b>200 mL</b>
0.2 M $K_2HPO_4$ - $KH_2PO_4$ pH 7	100 mL
0.1 M $MgSO_4$ ,	2 mL
1 M KCl	2 mL
1% Triton X-100	2 mL
1% N-laurylsarcosine	2 mL
Water	92 mL

### **β-gal reaction buffer**

Z buffer containing 5 mM  $K_3Fe(CN)_6$ , 5 mM  $K_4Fe(CN)_6$ , 0.1% Triton X-100, 0.1% N-laurylsarcosine and 0.8 mg mL<sup>-1</sup> M-gal.

<b>To prepare, just before use</b>	<b>10 mL</b>	<b>25 mL</b>
0.2 M $K_2HPO_4$ - $KH_2PO_4$ pH 7	5 mL	12.5 mL
0.1 M $MgSO_4$ ,	0.1 mL	0.25 mL
1 M KCl	0.1 mL	0.25 mL
1% Triton X-100	1 mL	2.50 mL
1% N-laurylsarcosine	1 mL	2.50 mL
0.1 M $K_3Fe(CN)_6$	0.5 mL	1.25 mL
0.1 M $K_4Fe(CN)_6$	0.5 mL	1.25 mL
40 mg mL <sup>-1</sup> M-gal	0.2 mL	0.50 mL
Water	1.6 mL	4 mL

## **PROCEDURES**

### **GUS fixation**

Submerge freshly cut roots in cold GUS fixative. Do not let methanol to warm up. Place roots immediately at -20°C for 2 h. Alternatively, roots may be kept in water during sample collection. Dry them gently with a tissue paper before submerging in fixative.

### **Washes**

Discard fixative and submerge roots in a generous volume of Pi-Ferri buffer. Incubate by gently agitation for 10 min. Discard solution and add fresh buffer, incubate as described. Repeat washing for a third time. These steps are carried out at room temperature.

### **GUS reaction**

Submerge roots in GUS reaction buffer. Infiltration in vacuum (20-30 min) will speed up the reaction by forcing penetration of the substrate, but will not have an appreciable effect for incubations longer than 4 h at 37°C. The reaction should be monitored to avoid excess of deposition of ClBr-indigo. A too dark blue appearance of plant cells interferes with the observation of the red precipitate product of M-gal.

### **Ending GUS reaction**

Submerge roots in washing buffer (0.1 M  $Na_2HPO_4$ - $NaH_2PO_4$  pH7.4), proceeding as described in the washing step. At this stage, roots can be examined and pieces of interest for staining of β-gal activity can be sectioned.

## Inactivation of endogenous plant $\beta$ -galactosidases

Place roots in endogenous  $\beta$ -gal inactivation buffer, infiltrate in vacuum for 30 min and let stand for 2 to 4 h at room temperature.

## Equilibration in Z buffer

Roots are incubated as described above in Z buffer.

## $\beta$ -gal reaction

Roots are submerged in freshly prepared  $\beta$ -gal reaction buffer and infiltrated for 30 min in vacuum. The vials are placed at 28°C for the reaction to take place. The reaction time should be carefully monitored, to avoid over staining.

## Ending $\beta$ -gal reaction

Discard reaction buffer and submerge roots in deionised water, for at least 1 h. Storing the roots overnight at 4°C will enhance deposition of product.

## Long-term preservation of double-stained tissue

Root pieces are post-fixed in 2.5% glutaraldehyde in 0.1 M Na<sub>2</sub>HPO<sub>4</sub>-NaH<sub>2</sub>PO<sub>4</sub> pH 7.4 for 4 h at room temperature or overnight at 4°C. Root pieces are rinsed in deionized water for 30 min and dehydrated by subsequent incubations (20 min per step) in 20, 50, and 70% ethanol. Roots can be stored for many months in 70% ethanol at room temperature or at 4°C.

## RESULTS

Observations appearing in Figure 1 were made using a Leica MZ12 stereoscopic microscope; pictures were taken with a Sony DKC-5000 digital camera. Roots have been cleared by submersion in chlorallactophenol (Beekman and Engler, 1994), with exception of the root appearing in panel D. In panels A, B, and D, rhizobia are easily detected in infection threads in root hairs. It seems that infection threads did not (yet) penetrate the root cortex. MsENOD12B is expressed in cell layers close to the pericycle in a rather long lateral root (panel A) and in an emerging lateral root, where is also strongly expressed at the tip of the root (panel B). Similar observations have been reported for MsENOD12A expression in roots of transgenic *M. sativa* ssp *varia* (Bauer et al, 1996) and MsENOD12B in transgenic *M. truncatula* roots (Trinh et al, 1998). In panel C, MsENOD12B seems to be induced in internal cortical cells below an extensive zone of infection thread formation, which have already penetrated external cortex cells. Cells expressing MsENOD12B cells correspond to rapidly dividing internal cortical cells that lead to the formation of nodule primordium in *L. japonicus* (Spronsen et al, 2001) Similarly, induction of

MsENOD12A in dividing cortical cells triggered by inoculation of *M. meliloti* Nod factors and cytokinins has been observed in transgenic *M. sativa* ssp *varia* roots (Bauer et al, 1996). Note that the gene is expressed in the cortex only at loci of infection, as reported for pea (Scheres et al, 1990) and *M. truncatula* ENOD12 genes (Pichon et al, 1994b).

In panel D, MsENOD12B is strongly expressed in a young, emerging nodule in *L. japonicus* roots, as has been observed for *M. truncatula* roots (Trinh et al. 1998).

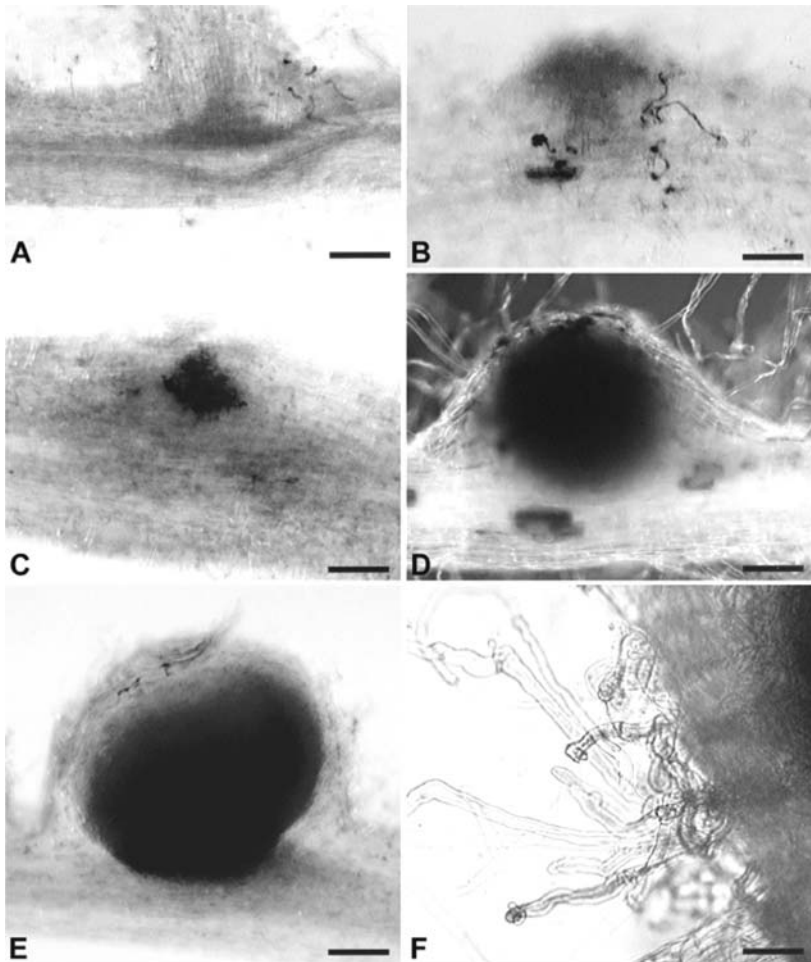


Figure 1. Double staining of *L. japonicus* hairy roots expressing MsENOD12B-*gusA* (*X*-gluc as substrate, blue precipitate) during nodulation induced by *M. loti* R7A constitutively expressing  $\beta$ -gal (*M*-gal as substrate, red precipitate). Bar in panels A, B, D, and E=125  $\mu$ m. Bar in panel C=95  $\mu$ m. Bar in panel F=30  $\mu$ m. See colour plate 3 (A-F) for this figure in colour.



Bacteria in infection threads in the epidermis can be seen by the red M-gal precipitate.

In panel E, MsENOD12B expression can be clearly observed at the base of a young nodule. Sectioning of embedded nodules is necessary for the precise localization of GUS expression and rhizobia inside nodules such as the ones presented in panels D and E. In panel F, a close-up of a root hair carrying an infection thread and deformed roots hairs observed on the surface of a mature nodule, show that *MsENOD12B* is induced in root hairs interacting with rhizobia. GUS activity was also observed in the root elongation zone (close to root tips) and the growing root hair zone of some lateral roots in plants inoculated by with *M. loti*, including root hairs (not shown). Taken together, these observations suggest that *MsENOD12B* share similar patters of expression in *L. japonicus* roots as the other *ENOD12* genes of plants that have an indeterminate type of nodulation.

## COMMENTS

### Solutions used for the detection of promoter-reporter gene activity

Freshly prepared solutions should be used to avoid bacterial contamination that could give rise to artefacts of staining. The pH of these solutions has to be checked before use, as the pH tends to fluctuate by salt precipitation after sterilization and long time storage of stock solutions.

### GUS-staining

Cytoplasmic protein precipitation in cold methanol is a very convenient way to fix *gusA*-expressing cells: we have not observed leakage of product even after long incubation time (overnight) of strongly expressed promoters. GUS expression of methanol-fixed roots compared well with that of paraformaldehyde-fixed roots. We have also observed that methanol fixation increases the permeability of *L. japonicus* root cells to glutaraldehyde and enzyme substrates, even if detergents are omitted from the incubation solutions used for endogenous  $\beta$ -galactosidases inactivation and reporter gene detection.

### Endogenous $\beta$ -galactosidases inactivation

We often found out that blocking of background staining of endogenous  $\beta$ -galactosidases in *L. japonicus* roots was not predictable, even if incubation time in inactivation buffer was extended overnight. This was particularly true when roots of old plants (4 to 8 weeks) were used for staining. The combination of methanol fixation and addition of detergents to the inactivation buffer resulted in complete blocking of background staining in the roots of plants up to 4 weeks old, and in considerable background reduction in the roots of older plants. In general, inactivation of endogenous  $\beta$ -galactosidases in older roots and mature nodules requires longer incubation times.

## Staining for $\beta$ -galactosidase activity in rhizobia

We have observed that storage of roots after inactivation of endogenous  $\beta$ -galactosidases for 48 h at 4°C does not seem to affect  $\beta$ -galactosidase activity encoded by *lacZ* pXLGD4. The stability of this reporter gene allows time for careful examination of GUS-stained roots before proceeding with the detection of bacterial  $\beta$ -galactosidase activity. During staining of the second reporter gene, roots can be inspected, pictures made, and the reaction continued by placing the roots back in the reaction buffer and incubating at 28°C. Alternatively, the reaction can be slowed down by storage at 4°C overnight, and continued the following day if necessary.

## Long-term storage of double stained tissue

As a standard, we perform post-fixation and store *L. japonicus* roots in 70% ethanol. Apart for ensuring aseptic conditions of storage, ethanol extracts many colouring substances from Lotus roots. Taking pictures and picking of root pieces and nodules for embedding can be made in 70% ethanol.

## Photography

Although many observations can be made directly on roots kept in 70% ethanol (see fig. 1D), detailed observations like bacteria in infections threads is greatly facilitated in cleared tissue. We have applied different recipes for the clearing of *L. japonicus* roots and we have found out that only submersion in chlorallactophenol (chloralhydrate: lactic acid: phenol 2:1:1, by weight, Beeckman and Engler, 1994) results in totally cleared roots. This solution is a fixative by itself; however, it tends to dissolve the red M-gal precipitate of freshly stained roots. Therefore, we take post-fixed roots that have been kept in 70% ethanol to 50 and 30% ethanol (20 min each) before incubation in three changes of water (at least 1 h each change) to ensure that ethanol residues have been washed away.

Roots are completely cleared after overnight incubation in clearing solution at room temperature; they can be stored for long periods in this solution in a tightly closed glass vial. Because chlorallactophenol is highly toxic and produces skin burns, only roots that have been carefully examined to address a particular detail are cleared. For mounting cleared roots, we use microscope slides that are prepared by fixing two cover slides of 20x20 mm on both ends of a slide with a contact epoxy resin, to prepare a chamber where one or two root pieces can be contained. The prepared slide is placed as a bridge on top of two slides positioned about 1.5 cm apart on the interior surface of a lid of a plastic Petri dish (diameter 9.5 cm) to its facilitate picking up. Root pieces are transferred on a drop of clearing solution to the middle of the chamber and the Petri dish is closed.

Roots can be screened in closed Petri dishes using a stereoscopic microscope. A cover slide is positioned on top of the roots before final observation and photographing; mounted slides can be kept for a few days at 4°C in Petri dishes

closed with Parafilm. The mounted glass slide can be easily picked up from the glass bridge and placed on a clean glass or plastic surface that can protect the microscope stage from contamination with clearing solution. We recommend that adjustment of the (digital) camera and the position or type of light source should be prepared with the specimen contained in a closed Petri dish as to restrict exposure to the fumes of chlorallactophenol to the shortest time possible. Mounting of cleared roots is carried out in a fume cabinet, using forceps and wearing gloves.

Chlorallactophenol is prepared by mixing 200 g chloral hydrate, 100 g lactic acid and 100 g solid phenol in a beaker placed in a water-bath at 60°C, in a fume cabinet. Transfer solution to a dark glass bottle that can be tightly capped to avoid oxidative browning.

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