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Auxin distribution in *Lotus japonicus* during root nodule development

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Abstract

For this work, *Lotus japonicus* transgenic plants were constructed expressing a fusion reporter gene consisting of the genes *β-glucuronidase* (*gus*) and *green fluorescent protein* (*gfp*) under control of the soybean auxin-responsive promoter GH3. These plants expressed GUS and GFP in the vascular bundle of shoots, roots and leafs. Root sections showed that in mature parts of the roots GUS is mainly expressed in phloem and vascular parenchyma of the vascular cylinder. By detecting GUS activity, we describe the auxin distribution pattern in the root of the determinate nodulating legume *L. japonicus* during the development of nodulation and also after inoculation with purified Nod factors, *N*-naphthylphthalamic acid (NPA) and indoleacetic acid (IAA). Differently than white clover, which forms indeterminate nodules, *L. japonicus* presented a strong GUS activity at the dividing outer cortical cells during the first nodule cell divisions. This suggests different auxin distribution pattern between the determinate and indeterminate nodulating legumes that may be responsible of the differences in nodule development between these groups. By measuring of the GFP fluorescence expressed 21 days after treatment with Nod factors or bacteria we were able to quantify the differences in GH3 expression levels in single living roots. In order to correlate these data with auxin transport capacity we measured the auxin transport levels by a previously described radioactive method. At 48 h after inoculation with Nod factors, auxin transport showed to be increased in the middle root segment. The results obtained indicate that *L. japonicus* transformed lines expressing the GFP and GUS reporters under the control of the GH3 promoter are suitable for the study of auxin distribution in this legume.

Abbreviations: CLSM, confocal laser scanning microscopy; dpi, days after inoculation; GFP, green fluorescent protein; GUS, β-glucuronidase; hpi, hours after inoculation; IAA, indoleacetic acid; NPA, *N*-naphthylphthalamic acid; Nod factors, nodulation factors

Introduction

Leguminous plants are able to have a mutualistic symbiosis with several genera of bacteria of the family Rhizobiaceae under nitrogen-limiting conditions. This relationship starts with signal molecule interchange between plant and bacteria that results in the formation of specialised plant organs called nodules on the plant root (Dénarié *et al*., 1996; Spaink, 2000). One of the first detectable modifications during the formation of the nodule are differences in calcium distribution at the cell membrane of the root hair followed by root hair curling, and initiation of organized cell divisions in the root cortex. These processes are induced by specific rhizobial signal molecules called Nod factors that are secreted in response to plant signal molecules. The latter are flavonoids and isoflavonoids exuded by the plant root (Kijne, 1992; Dénarié *et al*., 1996; Spaink, 2000).

Both plant flavonoids and microbial Nod factors have been reported to be directly involved in regulation of the distribution of indoleacetic acid (IAA),

the natural auxin in plants. Rhizobial Nod factors are thought to drive the formation of the root nodule through manipulation of the plant IAA levels (Jacobs and Rubery, 1988; Mathesius *et al*., 1998; Mathesius, 2001).

Auxin is not uniformly distributed in the plant. The highest concentrations are found in the plant cells undergoing cell divisions, elongation and re-directioning during tropisms like gravitropism and phototropism (Rinhardt *et al*., 2000). The exact mechanism of auxin signalling, transport and regulation is still not completely understood (for a review, see DeLong *et al*., 2002).

Auxin is produced in the plant shoot and moves down to the root tip following an auxin concentration gradient between upper and lower cells of the xylem parenchyma with the help of auxin efflux protein complexes (Lomax *et al*., 1995; Jones, 1998). The concentration of auxin at different sites of the plant may be regulated by the distribution of the auxin efflux proteins. These proteins are not symmetrically distributed along the cell membrane and are subject to dynamic reallocation (Geldner *et al*., 2001). Different compounds like NPA or tri-indobenzoic acid (TIBA) can inhibit the acropetal auxin transport. NPA is thought to bind to the auxin transporters present at the basis of the cell, preventing the auxin flux to the adjacent cells. Several classes of flavonoids have a similar effect on auxin transport as NPA in *Trifolium repens* (white clover) and non-leguminous plants like *Arabidopsis thaliana* (Jacobs and Rubery, 1988; Mathesius, 2001, Brown *et al*., 2001). Similarly, Nod factors inhibit the acropetal auxin transport in roots of white clover and in *Vicia sativa* (vetch) (Mathesius *et al*., 1998; Boot *et al*., 1999; Mathesius, 2001).

The use of the auxin-regulated soybean promoter GH3 (Hagen *et al*., 1991) fused to the reporter gene *gusA* has yielded new insights into auxin distribution and expression in, among others, leguminous plants. In GH3 expression studies it has been observed that this promoter is active only in tissues where auxin is present at high levels. Furthermore, this promoter has a quick response to auxin concentration variations (Larkin *et al*., 1996; Mathesius *et al*., 1998; Li *et al*., 1999; this work). After gravistimulation of white clover plants transformed with a GH3:*gusA* fusion it was observed that GUS expression was enhanced in an asymmetric way to the point where the root curvature appeared. In the nodules and lateral roots the GUS expression was present at dividing cells (Larkin *et al*., 1996). In the same transgenic lines,

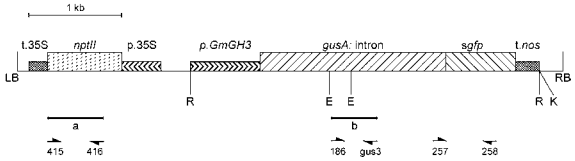

Figure 1. T-DNAs used to transform *L. japonicus* plants and primers used in analysis. The binary vector pMP3632 contains the bifunctional *gus:intron/gfp* gene driven by the auxin-inducible promoter GH3 from *Glycine max* and the *nos* terminator in pPZP111 in the orientation as indicated. Primers used in PCR and probes used in Southern hybridization are depicted in the lower part of the figure. Restriction sites: R, *Eco*RI; E, *Eco*RV; K, *Kpn*I. Only relevant restriction sites are indicated. Abbreviations: LB, left border; p, promoter; RB, right border; t, terminator. The direction of transcription is indicated by arrows in the promoter regions.

at early stages of nodulation, auxin was acropetally down-regulated from the point where the nodulating bacteria (*Rhizobium leguminosarum* bv. *trifolii*) were inoculated, and up-regulated at the site where the nodule started its development. The same effects were found after spot inoculation of Nod factors produced by the same bacterial strain, and external application of NPA (Mathesius *et al*., 1998).

L. japonicus is used as a model legume because of its easy and rapid growth under laboratory conditions, a high level of seed production, self-fertility, a small genome and, most importantly, because it can be easily transformed by *Agrobacterium tumefaciens* resulting in stable transfomants (Handberg and Stougaard, 1992; Jiang and Gresshoff, 1997; Szczyglowski *et al*., 1998).

The fusion protein GUS/GFP has been reported to be a promising marker for study in *L. japonicus* (Quaedvlieg *et al*., 1998). For our work stable *L. japonicus* transformants expressing a *gusA/gfp* fusion gene under control of the soybean GH3 promoter were made and a combined analysis of the GH3 promoter activity was performed by means of histochemical staining for GUS activity and GFP fluorescence detection in living plants. We also were able to make a quantitative analysis of GH3 expression levels during nodule primordia formation and in mature nodules by CLSM analysis of GFP fluorescence. We show that *L. japonicus*, which belongs to the determinate nodulating legume group, differs in GH3 expression from white clover, which forms indeterminate nodules.

Material and methods

Plasmids and bacterial strains

The soybean GH3 promoter (p*Gm*GH3) (Hagen *et al*., 1991) was isolated from pJJ430 (Larkin *et al*., 1996) and fused to the 5′ end of the *gusA:intron/gfp* fusion gene in pMP2168 (Quaedvlieg *et al*., 1998) yielding pMP3631. The binary vector pMP3632 (Figure 1) resulted from cloning of an *Eco*RI fragment from pMP3631 containing the *gusA:intron/gfp* fusion gene under control of the *Gm*GH3 promoter and the nopaline synthase (*nos*) terminator sequence into *Eco*RI-digested pPZP111 (Hajdukiewicz *et al*., 1994). Plasmids were transferred to *A. tumefaciens* strain LBA4404 (Hoekema *et al*., 1984) by triparental matting with pRK2018 as mobilizing plasmid (Ditta *et al*., 1980). Transformed bacteria were selected on LB medium (Sambrook *et al*., 1989) supplemented with rifampicin (20 μg/ml) and chloramphenicol (10 μg/ml).

Plant transformation, selection and analysis of transgenic plants

L. japonicus ecotype GIFU was transformed with pMP3632 and pPZP111 by the *A. tumefaciens*-mediated hypocotyl transformation protocol according to Handberg *et al.* (1992) and slightly modified as described by Quaedvlieg *et al.* (1998). Transgenic lines were selected with G418 (25 μg/ml). Antibiotics were omitted at the root induction and root elongation steps.

Young primary transformed plants (T_1) were analysed for the presence of transgenes by PCR with Super *Taq* polymerase according to the manufacturers' protocol (HT Biotechnology, Cambridge, UK). Template DNA was obtained by a quick isolation protocol (Wang *et al*., 1993). The following specific primer sets were used in separate reactions: oMP257 (5′-GGGAGCTGTTCACCGGGGTGG-3′) with oMP258 (5′-GCTGCACGCTGCCGTCCTCG-3′) for *gfp* and oMP415(5′-ACCGTAAAGCACGAGGAAGC-3′) with oMP416 (5′-TTGTCAAGACCGACCTGTCC-3′) for *nptII*. The primer set oMP316 (5′-ACCTACTC-ATCTGCAGAAAC-3′) and oMP317 (5′-GAGCTC CATGGTTACCAGATTC-3′) was used as internal control to detect the *Lj*ENOD40-1 gene. A selected number of primary transformed plants was analysed by Southern hybridization. Total DNA was isolated from leaves, digested either with *Eco*RI, *Eco*RV or *Kpn*I (Figure 1) and separated by gel electrophoresis. Gels also contained lanes loaded with digested purified plasmid DNA of pMP3632, digested total *L. japonicus* DNA of wild-type plants and lines stably transformed with either pPZP111 or pMP2182 (Quaedvlieg *et al*., 1998). Genomic plant DNA was isolated with the Nucleon PhytoPure Genomic DNA extraction kit of Amersham Biosciences (Roosendaal, Netherlands), digested with *Eco*RI, *Eco*RV or *Kpn*I, separated by gel electrophoresis, and blotted onto positively charged membranes (Roche, Mijdrecht, Netherlands). Southern hybridization reactions were performed with the non-radioactive dioxygenin (DIG) labelling and detection kit of Roche according to the manufacturers' instructions (Roche). Both *gusA* and *nptII* DIG-labelled probes were obtained by PCR with the primer sets oMP186 (5′-GTCGCGCAAGACTGTAACCACGCGTCTG-3′) with Φ gus3 (5′-GCCTAAAGAGAGGTTAAAGCC-3′) and oMP415 with oMP416, respectively (Figure 1). Each restriction sample was split into halves and loaded on two gels to obtain identical blots, which were treated with the *gusA* and *nptII* probes, respectively. Hybridizing bands were visualized by exposure to Fuji X-Ray films.

Various parts of primary transformed plants were analysed for GUS and GFP activity by histochemical staining and fluorescent microscopy, respectively, as described below. In later generations GFP expression was detected by confocal laser scanning microscopy (CLSM). A set of 112 lines in the T_2 generation was generated from a selected number of T_1 lines. From these plants, seeds were analysed for germination efficiency and GUS staining rates of the seedlings. A detailed description of the analysis of all these T_2 lines can be found on the Internet: http://rulbim.leidenuniv.nl/publications/PMB_Pacios.htm. The lines presenting homogeneous GUS expression and a high germination rate were also checked for GFP expression by CLSM.

Histochemical staining for GUS activity

GUS activity was determined histochemically by immersion of the plant material in 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc) solution in 50 mM sodium phosphate buffer pH 7.2, 0.1% Triton X-100, 10 mM EDTA, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$ at 37 °C in darkness and cleared in 70% ethanol before visualization. Plant material was incubated for 45 min without application of vacuum. GUS expression was examined with a Leica MZ12 stereo microscope and images recorded with a Sony

DKC5000 digital camera and processed with Adobe Photoshop 5 software.

GFP detection and quantification

GFP fluorescence was detected in primary transformed plants (T_1 generation) with a Leica fluorescent stereo microscope and in T_2 plants with a Leica SP CLSM. GFP was excited at 488 nm and detected at 500–550 nm. For CLSM 5 μm xy optical sections were made of the samples. For quantification the middle section of each series of optical sections was chosen. The GFP of an area between 4700 and 4800 μm^2 was quantified by the use of the quantification option of the microscope software (Leica TCSNT). The average values of each measured point were used for evaluation of GFP intensity at different heights of the root. The images obtained were further processed by using ImageJ version 1.12 and Adobe Photoshop 5 software.

Isolation and analysis of Nod factors

Nod factors were isolated from 1 litre of *M. loti* strain R7A culture as described (López-Lara *et al*., 1995) and dissolved in 5 ml 60% acetonitrile (ACN)/water. The equivalent to 600 ml culture in 6 ml 30% ACN/water was submitted to HPLC with the same increasing ACN/water gradient as described by Pacios-Bras *et al*. (2002). The fractions with absorption at 206 nm were collected, dried and dissolved in dimethyl sulfoxide (DMSO) to an approximate final concentration of 10^{-3} M, calculated by comparison with the Nod factors concentrations published for *M. loti* strain ER1 (López-Lara *et al*., 1995). The sample was submitted to ion trap mass spectrometric analysis as described by Pacios-Bras *et al*. (2002).

Plant growth and inoculation

L. japonicus transformed plants were grown on vertical dishes containing Jensen medium (van Brussel *et al*., 1986) supplemented with L-α-aminoethoxyvinylglycine (AVG) and with the roots shielded from light (Pacios Bras *et al*., 2000). All the plants were grown in a 21 °C acclimated chamber with a 16 h light/8 h dark photoperiod.

Spot inoculation was done by the application of sand grains coated with the compound of interest (López-Lara *et al*., 1995) on 2-day old *L. japonicus* roots. A volume of 40 μl 10 mM NPA in DMSO, 20 μl Nod factors 10^{-3} M in DMSO or 40 μl 1 mM IAA in 96% ethanol were mixed with 60% ACN/water to a final volume of 1.5 ml and incubated overnight at room temperature with 100 mg sand. For mock inoculations 100 mg sand was treated in the same way with 1.5 ml 60% ACN/water. Subsequently the samples were dried in a Centrivap concentrator (Labconco, Kansas City, MO). One or two grains of coated sand were used for the spot inoculation of each plant. For some experiments, 2 μl of 10^{-3} M Nod factors in 60% ACN/water were directly inoculated on the root. For these experiments control roots were inoculated the same way with 2 μl 60% ACN/water. For nodulation experiments 200 μl *M. loti* R7A aqueous suspension (OD_{660} 0.1) was inoculated to each plate, containing 8 to 10 two-day old seedlings each.

For GFP analysis (21 dpi) the plants were grown on petri dishes as described above, decapitated before visualization with CLSM and immediately after analysis stained for GUS following the described protocol.

Localization of GUS activity

Roots of control transgenic plants were mock inoculated with ACN-treated sand and stained for GUS expression 48 hpi, were chopped in ca. 3 mm segments which were fixed for 2 h in 2.5% glutaraldehyde/2% paraformaldehyde in 0.1 M phosphate buffer pH 7.0. After rinsing in phosphate buffer the samples were dehydrated by immersion into consecutive series of increasing concentrations of ethanol/water (70%, 80%, 96% and 100% ethanol) in steps of 30 min each. The final step was performed for 45 min. Next, the root fragments were embedded in Technovit 7100 (Kulzer, Wehrheim, Germany) following the manufacturers' instructions. Sections of 5 μm were made with a LKB pyramitome (Bromma, Sweden), and stained with a 0.5 safranin/water solution for 5 min at room temperature. A Zeiss axioplan light microscope was used for visualization, images were recorded with a Sony DKC-5000 digital camera and processed with Adobe Photoshop 5 software.

Auxin transport assay

Two-day old plantlets of the T_2 generation lines *Lj*3632.5.127 and *Lj*3632.5.126 were inoculated with 2 μl Nod factors in 60% ACN or with 2 μl 60% ACN/water (control plants) and grown under the conditions described above. Auxin transport measurements were done 24 hpi, 48 hpi and 21 dpi respectively. For these experiments a segment of 16 mm

length comprising the lower and middle part of the root without the root tip of 4 mm was excised from the shoot of each plant. The upper end of the root fraction was incubated in agar containing 10^{-7} M labelled IAA (^{3}H-IAA) (Amersham) and AVG at a final concentration of 0.1 mg/l for 3 h as described (Boot *et al*., 1999). Next the root fragment was divided into three segments, two basipetal ones of 4 mm each and the one most distal from the root tip at 8 mm. ^{3}H-IAA transport by the middle 4 mm root segments was measured at each time point after inoculation for 72 h with a LKB Wallac1214 Rackbeta liquid scintillation counter. The same measurements were performed with the control ACN/water-inoculated plants.

Results

Generation and analysis of transgenic L. japonicus

Hypocotyl cuttings of *L. japonicus* were transformed with pMP3632 (Figure 1) and pPZP111 (control) both showing similar transformation efficiencies. In total, 64 independent primary transformed lines (T_1 generation) were regenerated from G418-resistent calluses that resulted from transformation with pMP3632. From the control, only a few plants were regenerated. The T_1 generation of pMP3632-transformed plants was analysed by PCR, Southern hybridization and detection of GUS enzymatic activity of the bifunctional *gus:intron/gfp* reporter gene (Quaedvlieg *et al*., 1998) before seed set. Small pieces of leaves of independently regenerated pMP3632-transformed plant lines were analysed by PCR before they were transferred to soil. As controls, material was taken from wild-type *L. japonicus* and from plants stably transformed with pPG1 or pMP2182 harbouring the *nptII* gene and the reporter gene *gus:intron* or *gus:intron/gfp*, respectively, under control of the 35S promoter from CaMV (Quaedvlieg *et al*., 1998). Only 41 of the 46 pMP3632-transformed plants analysed (89%) demonstrated the presence of the *nptII*-containing PCR fragment (592 bp) whereas in the control samples (pPG1- and pMP2182-transformed material) a fragment could always be amplified. Specific primers for the *gfp* gene were used to determine whether the gene of interest was present. From 35 plant lines (95%) a PCR fragment could be amplified of the same size (517 bp) as was observed for the control containing the same reporter gene (pMP2182). Combining the results, it was shown that 2 plant lines of the 46 lines analysed (4%) lacked both the *nptII* and the *gfp* gene indicating they were false-positive regenerated plants. Two plant lines (4.3%) likely harbour a deletion at the right-border site of the T-DNA since from these a *nptII* fragment could be amplified but not a *gfp* fragment. No plant lines were observed that were positive for *gfp* and negative for *nptII* as shown by PCR indicating that deletions at the left-border site of the T-DNA were not present in our collection of primary transformed plants.

Twenty-two independent lines of pMP3632-transformed plants that showed satisfactory PCR results and were growing well in soil were subjected to Southern hybridization analysis (data not shown). Control DNA from wild-type plants never showed hybridizing bands indicating that no background hybridization occurred with the probes used. Eight plant lines were found to have a single insertion of pMP3632 (lines *Lj*3632.5, *Lj*3632.8, *Lj*3632.28, *Lj*3632.34, *Lj*3632.40, *Lj*3632.43, *Lj*3632.55 and *Lj*3632.57) as judged from a single hybridizing band with either probe.

Well developed, flowering primary transformed plants growing in soil were assayed for activity of the reporter gene as it was expected that at least in some tissues the *Gm*GH3 promoter would be constitutively active (Mathesius *et al*., 1998). In different organs from 60 independent lines GUS activity was determined histochemically. To ensure the detection of low levels of expression as well, relatively high levels of the substrate X-Gluc were used. Plant material was subjected to vacuum infiltration and a long incubation time. After clearance, plant parts were analysed in detail under a binocular microscope. Blue staining was observed in 29 plant lines (48%). Large variations were observed in intensity of the staining as well as in organ or tissue specificity. Plant lines containing a single transgenic insert always showed GUS expression usually at high levels. Contrary, transgenic lines harbouring multiple inserts often did not show GUS activity or only in some tissue. For instance, line *Lj*3632.20 showed GUS activity in the vascular bundle of the root but not in that of the stem. The expression of GFP was assayed in different organs of 19 transformed lines. Of 13 lines (68%), plant parts were considered to have a specific GFP expression. All of these lines did show a high GUS activity as well.

Selection of transgenic lines for further study

The transgenic T_1 lines *Lj*3632.5, *Lj*3632.34, *Lj*3632.40, *Lj*3632.43, *Lj*3632.55 and *Lj*3632.59, all

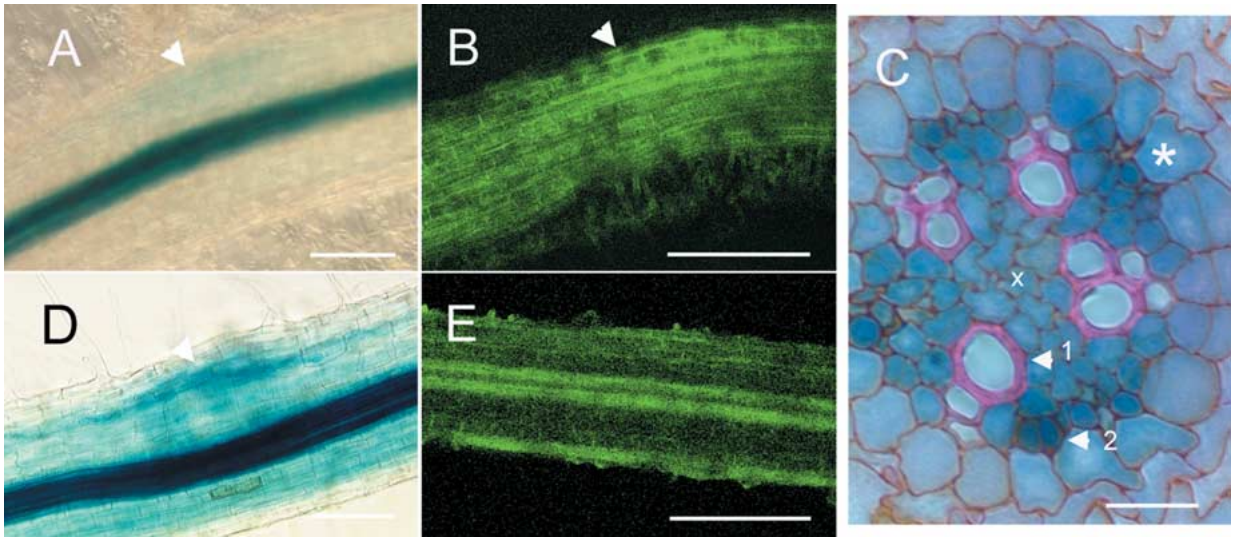

Figure 2. GUS (A and D) or GFP (B and D) expression of *Lj3632.5.127* plants. At the spots were actively dividing or elongating cells were present, a cortical blue staining in the case of GUS activity (A and D) or a green fluorescent signal in the case of GFP (B) was detected. A, B and E. Untreated plants. D. Cortical cell divisions leading to the formation of a nodule after inoculation with *M. loti* R7A. C. Transversal section of 5 μm of a mature part of a mock-inoculated root of line *Lj3632.5.127.* The roots were incubated at 48 hpi in X-Gluc and, after sectioning, stained with safranin as described in Materials and methods. Blue staining was mostly found at the phloem cells of the vascular bundle (arrowhead 2), in the parenchyma and, at lower levels, at the pericycle (*) and vascular parenchyma (+). Xylem is indicated with arrowhead 1. Scale bars 150 μm.

harbouring a single insert of the T-DNA and presenting the best phenotypes, were selected for bulk seed generation. From the selected primary transformants 112 T_2 lines were obtained and screened for seed germination rate and GUS expression of the T_3 offspring to determine whether the lines are homozygous or heterozygous. As in the T_1 generation, GUS expression, detected as blue staining, was variable in intensity along seedlings from different lines, but constant in seedlings pertaining to a single line. Analysis of GUS activity of T_3 lines revealed that 7 of the 28 T_2 descendent lines from *Lj*3632.5 were homozygous for GUS expression (100% of the T_3 seedlings showing GUS activity); 14 of the T_2 lines were heterozygous for GUS expression (having only part of the T_3 population expressing GUS). In the remaining 7 T_2 descendant lines GUS staining was absent.

Based on their homogeneous and homozygotic GUS expression, together with their high germination efficiency, lines *Lj*3632.5.126, *Lj*3632.5.127, *Lj*3632.34.120, *Lj*3632.59.109 and *Lj*3632.59.111 were selected for nodulation experiments with *M. loti*. Line *Lj*3632.34.120 had a regular but weak GUS expression in the root (data not shown). This phenotype was used to ensure that high expression levels of GUS in the other lines did not influence detection of auxin level changes. The other lines presented strong GUS expression. The selected lines were also checked for GFP expression. Only line *Lj*3632.5.127, in addition to high GUS expression, presented a high and homogeneous GFP expression which was clearly discernible when analysed by CLSM (Figure 2B and E). This line was used for *in vivo* analysis. In the rest of the lines analysed, the specific signal for GFP was not detectable above autofluoresence.

GUS expression in untreated plants

Untreated plants of the selected lines from the T_2 generation (*Lj*3632.5.126, *Lj*3632.5.127, *Lj*3632.34.120, *Lj*3632.59.109 and *Lj*3632.59.111) exhibit a blue staining indicative for GUS activity in the root vascular bundle with an equal distribution. The staining was detected in vascular tissue of roots (all lines), leaves (*Lj*3632.5.127 and *Lj*3632.34.120) and flowers (determined for line *Lj*3632.5.127 and *Lj*3632.34.120). Additionally, in the cases that the root presented some curvature or cell divisions, standing out blue cells were detected in the root cortex (Figure 2A). Wild-type *L. japonicus* did not show any blue cells after incubation with X-Gluc indicating that the staining we observed was due to activity of the GUS enzyme encoded by the transgenes.

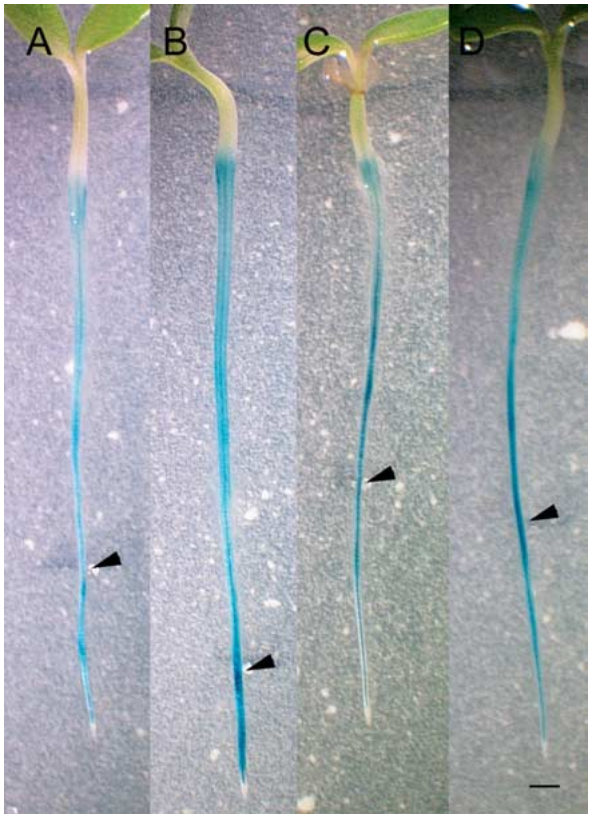

Figure 3. GUS expression pattern of *Lj3632.5.127* plants. A (top). 24 hpi after mock inoculation (control sand grains treated with ACN/water) (A) or inoculation with sand grains coated with IAA (B), NPA (C) and Nod factors (D). The inoculation site is indicated with an arrowhead. Scale bars 0.5 cm. B (stronger magnification, bottom). Left, 24 hpi after mock inoculation with uncoated sand grains; middle, inoculation with sand grains coated with IAA; right, inoculation with sand grains coated with LCOs.

After visualization of 5 μm sections of untreated plants of line *Lj*3632.5.127 we saw that in mature regions of the root, GUS expression was restricted to the phloem poles of the vascular cylinder. A lower level of expression could also be detected in the pericycle and vascular parenchyma (Figure 2C).

GUS expression after inoculation with IAA and NPA

L. japonicus transformants lines *Lj*3632.5.127 and *Lj*3632.34.120 were spot-inoculated with sand coated with IAA and NPA and analysed for GUS expression 6, 24 and 48 hpi (Figure 3). As control, mock-coated sand grains were used. Inoculation of IAA induced in all cases a clear thickening of the root and a large increase in root hair number and length. Already 6 hpi a strong GUS expression at the root tip of the IAA-inoculated plants was detected in 6 of 8 plants inoculated of line *Lj*3632.5.127. This effect was not seen in any of the 5 inoculated plants of line *Lj*3632.34.120. This up-regulation acropetal from the inoculated spot together with the root tip thickening was most clear 24 hpi (Figure 3B), being present in all of the plants inoculated (8 plants of line *Lj*3632.5.127 and 3 of line *Lj*3632.34.120). In these plants, the root segment acropetal to the spot inoculation site always remained shorter than in mock-inoculated plants. At 48 hpi the whole root presented a higher GUS staining than the control plants in 5 of the 8 inoculated roots of line *Lj*3632.5.127 and 2 of the 4 IAA-treated *Lj*3632.34.120 plants.

The effect of NPA spot inoculation was not as clearly defined as after inoculation with IAA. A slight up-regulation of GUS activity at the spot inoculation point was detectable 6 hpi in 2 of the 4 roots inoculated of line *Lj*3632.34.120 and in none of the 5 roots inoculated of line *Lj*3632.5.127. This effect was more clearly visible 24 hpi; at this time point a basipetal up-regulation of GUS activity was also detected and a down-regulation close to the root tip (Figure 3C). This effect was seen in 6 out of 8 plants of line *Lj*3632.5.127 and in 4 out of 5 plants of line *Lj*3632.34.120. At 48 hpi NPA induced a down-regulation of GUS expression acropetal from the inoculated site (7 plants out of 13 of line *Lj*3632.5.127 and in 1 plant out of 4 of line *Lj*3632.34.120).

Detection of GUS expression after inoculation with Nod factors

Nod factors produced by *M. loti* strain R7A were isolated and analysed by mass spectrometry. The result

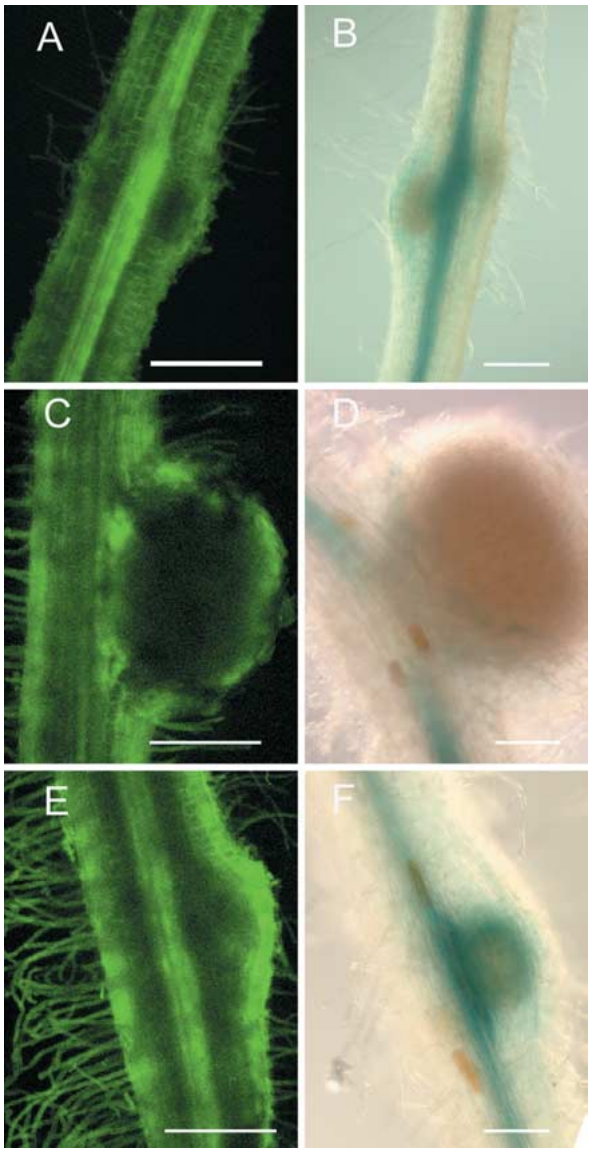

Figure 4. GUS (B,D, F) or GFP (A, C, E) expression on line *Lj3632.5.127* after inoculation with either *M. loti* R7A (C, D, E, F) or its Nod factors (A, B). A, B. Expression around nodule primordia induced 21 dpi after application of nod factors. C, D. Expression in mature nodules.

revealed that the structures were the same as those described before for *M. loti* strain ER1 (López-Lara *et al*., 1995).

Roots of lines *Lj*3632.5.126, *Lj*3632.5.127 and *Lj*3632.34.120 were inoculated with sand coated with Nod factors for analysis 6, 24 and 48 hpi or were directly inoculated with 2 μl Nod factor solution for analysis 21 dpi. Roots of control plants were inoculated in the same way omitting the Nod factors. At 6 hpi no clear differences were detected in the distribution of GUS activity as compared with control plants. The first differences in GUS expression were detected 24 hpi. At this time point a slight up-regulation was detected at the spot were Nod factors had been inoculated and a decrease in staining due to lower GUS activity was found at the root tip (Figure 3D). This effect was found in 8 of 8 inoculated roots from line *Lj*3632.5.127 and in 3 of the 4 roots inoculated of line *Lj*3632.34.120. After 48 h the down-regulation acropetal from the spot inoculation site was only detected in 4 roots of the 17 inoculated roots analysed. At 21 dpi most of the plants had formed nodule primordia (10 out of 12 plants in the case of *Lj*3632.5.126 and 7 out of 10 plants in line T2 *Lj*3632.5.127). The number of nodule primordia formed on a single root varied from 1 to 6. In several cases these plants showed lateral roots parting from the spot inoculation point (7 out of 12 plants in *Lj*3632.5.126). At the places where small nodule primordia were found a clear, more intense staining at the root cortex due to higher GUS activity was observed. The outer cortical layers of these primordia also showed blue staining (Figure 4B).

GUS expression after inoculation with M. loti

Several *L. japonicus* transgenic lines (*Lj*3632.55.3, *Lj*3632.59.11, *Lj*3632.5.126 and *Lj*3632.5.127) were inoculated with *M. loti*. The first blue staining linked to cortical cell divisions was detected between 2 and 5 dpi in all cases (Figure 2D). At 5 dpi some plants presented the first nodule primordia. During the primordia development, the blue staining was present at the ourter layers of the cortex and, like after inoculation with Nod factors, nodule primordia showed a higher GUS expression in the main root vascular directly in contact with the primordia (Figure 4F). This up-regulation disappeared when nodules developed (Figure 4D). In mature nodules, GUS expression was mainly present in the nodule vascular tissue and in some cases probably due to residual GUS activity, in a weak diffuse form, in the nodule cortex (data not shown).

Detection of GFP expression after inoculation with M. loti *or its purified Nod factors*

In untreated plants of line T2 *Lj*3632.5.127, GFP was detected in the root vascular bundle as two continuous parallel lines (Figure 2E), confirming the results obtained with GUS. At the positions in the root were elongation or cell divisions were taking place, a strong

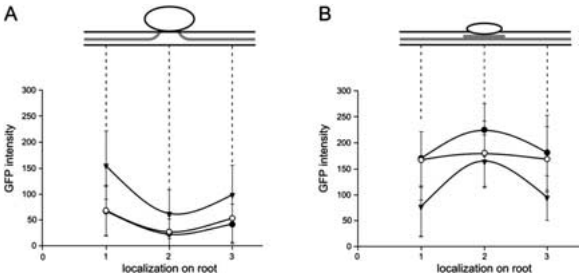

Figure 5. GFP quantification of *L. japonicus* roots of line *Lj3632.5.127* at 21 dpi inoculated with *M. loti* R7A (A) or Nod factors (B). Measurements were made at the root vascular bundle at the basis of the nodule or nodule primordia (2) and basipetal (1) and acropetal from the outgrowth (3). The distance between two measuring points is ca. 100 μm. In the basis of the nodule primordia (B) clearly higher GFP expression was detected which was inexistent in mature nodules (A). Each graphic (▲, ○, ●) represents the measurements on a nodule of a single plant.

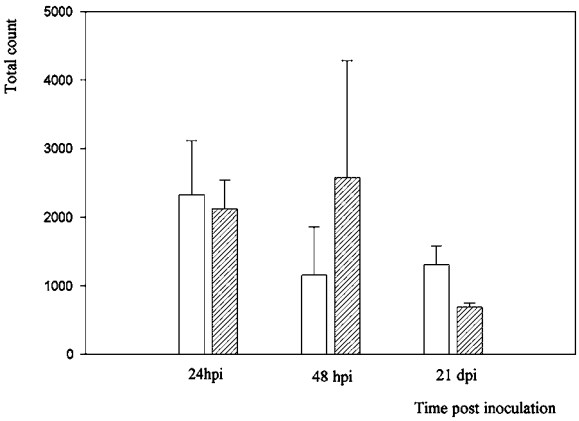

Figure 6. Quantification of the auxin transport in roots of line *Lj*3632.5.127 after inoculation with Nod factors at 24 hpi, 48 hpi and 21 dpi. The graphic shows a representative quantification of each time point after inoculation performed as explained in Materials and methods. White bars show the auxin transport of control roots while diagonally filled bars show the auxin transport of spot inoculated roots. This experiment was independently performed twice with either transformed or wild-type *L. japonicus* plants.

fluorescent signal was detected in the root cortex (Figure 2B). In the root tip the fluorescence was higher than in the rest of the root, probably due to the high amount of flavonoids and other autofluorescent compounds present at this position of the root. *L. japonicus* leaves presented high levels of red autofluorescence, which complicated GFP detection. Flowers were not analysed for GFP expression.

Differences in GFP expression in line *Lj*3632.5.127 were difficult to measure at the first time points after inoculation. At the time the first primordia were formed (15–20 dpi) quantification of GFP was feasible due to the presence of the nodule primordium as a reference point for CLSM visualization (Figure 5A).

Line *Lj*3632.5.127 was also used for study of GFP expression after inoculation with *M. loti* or the bacterial Nod factors. Like GUS activity, GFP expression was up-regulated in the main root at the position were the growing primordia were located (Figure 4E), this higher expression was absent in mature nodules (Figure 4C). The differences in GFP signal acropetal and basipetal from the mature nodule were quantified (Figure 5A). The results showed that GFP expression is down-regulated at the basis of the nodule and has a similar expression level immediately basipetal or acropetal of the nodule (Figure 5A).

The same regulation as described for GUS after inoculation with *M. loti* purified Nod factors was found when GFP was visualized. The vascular bundle of the root presented an upregulated segment at the position where the nodule primordium was developing (Figures 4A and 5B). GFP proved to be expressed at different levels in different roots of line *Lj*3632.5.127. This problem was circumvented by expressing the effect of Nod factors as a relative difference of the GFP signal present basipetal and acropetal of the nodule primordia. The results show that this ratio was always proportional in each root (Figure 5B).

Local auxin transport levels

Transport of ^{3}H-IAA in lines *Lj*3632.5.126 and *Lj*3632.5.127 was measured at 24 hpi, 48 hpi and 21 dpi with Nod factors. A 16 mm root segment was sectioned in three pieces as described in Materials and methods, and the ^{3}H-IAA level of the middle 4 mm fraction was quantified. The results showed that at 24 hpi no significant differences were detected between the plants inoculated with Nod factors or only treated with ACN/water. At 48 hpi with Nod factors a significant increase in auxin transport was detected in the same segment (Figure 6). The quantifications of ^{3}H-IAA at 21 dpi showed a lower concentration of ^{3}H-IAA in the plants treated with Nod factors than in the control plants. These results show that at 48 hpi with Nod factors, *L. japonicus* roots present a transitional up-regulation in auxin transport levels that is not present at 21 dpi.

Discussion

In this work we describe the auxin distribution pattern in the model legume *Lotus japonicus* in the presence of compounds that influence the natural distribution of IAA and during the course of nodulation. With this purpose, we made use of *L. japonicus* lines stably transformed with the *gus/gfp* reporter gene driven by the auxin-responsive promoter GH3. PCR was used as an easy and fast assay to analyse primary transformed plant lines for the presence of the transgenes. We demonstrate that *L. japonicu*s transformed plants present many advantages for the study of the auxin distribution in this species. Having lines with a homogeneous population facilitated the screening of the plants and considerably lowered the number of plants needed to achieve consistent results. Furthermore, the simultaneous use of two reporter proteins, GUS and GFP, permitted analysis of the same plants both fixed and *in vivo*. *L. japonicus* belongs to the so-called determinate-nodulating legumes. In these plants, the first cell divisions leading to nodule formation occur at the outer cortical layers and lead to round-shaped nodules with a limited growth (Szczyglowski *et al*., 1998). Before this study, the auxin distribution patterns of two other legumes, namely in white clover by the study of rooted leafs (Larkin *et al*., 1996; Mathesius *et al*., 1998) and *V. sativa* by the application of a split root system (Boot *et al*., 1999), have been described. These plant species form indeterminate-type nodules, meaning that the nodule cell divisions start at the root pericycle and the nodules are elongated with a continuously growing meristem.

Transformed *L. japonicus*, regenerated from seeds, showed similar changes in GH3 expression as white clover after application of IAA to the root. In both cases, auxin induces acropetal up-regulation from the inoculation point. In clover, microtargeting of IAA was necessary to see the effects whereas *L. japonicus* showed this response by external spot inoculation of the morphogen. Spot inoculation of NPA on the root showed in *L. japonicus*, like in white clover (Mathesius *et al*., 1998), an up-regulation at the spot inoculation site. In *L. japonicus* the dividing outer cortical root cells, leading to a nodule primordium, showed strong GUS expression. This expression pattern was not described for white clover (Mathesius *et al*., 1998). This result indicates that the local increase of auxin levels in the outer cortex of the root may be the plant determinant for the location of the first divisions of the nodule primordium in determinate-nodulating plants. We also observed an up-regulation of auxin levels at the basis of the nodule primordia induced by both rhizobia and purified Nod factors. This up-regulation was not present in mature nodules. A similar staining pattern was found in starting and already developed lateral roots. We also quantified the variation in auxin transport in *L. japonicus* roots after inoculation with purified Nod factors by the use of ^{3}H-IAA. The results showed a local up-regulation in auxin transport at a 4 mm segment 8 mm basipetal from the root tip which was not detected by GUS expression analysis. We do not know the correlation between auxin transport and auxin distribution in *L. japonicus* but, remarkably, the time at which the first root cortical cell divisions are detected after inoculation with *M. loti* (2 dpi) coincides with the moment in which the auxin transport is increased after inoculation with Nod factors. This probably indicates a link between the measured increase in auxin transport and a higher GUS activity in *L. japonicus* transformed lines at this time point.

Although we demonstrate here that the GH3 promoter was detectable simultaneously with both GFP and GUS reporter proteins, which is appropriate for the detection of differences in auxin distribution in *L. japonicus*, the detection of those changes might be improved by using the synthetic auxin-responsive promotor DR5, which has been shown to be activated only above a certain threshold (Sabatini *et al*., 1999). Furthermore, by making use of the GFP reporter, detection of changing expression level can be studied by the technique of fluorescent recovery after photobleaching (FRAP). This technique will provide more insight into the distribution and dynamics of auxin in *L. japonicus* in time.

The results obtained by such experiments may give us information about the different ways of plant root auxin distribution. For further understanding of the auxin distribution in *L. japonicus* it would be also interesting to analyse the auxin distribution after submission to gravitropism. The study of the auxin pattern after inoculation of heterologous rhizobia (Pacios Bras *et al*., 2000) and its Nod factors will be another interesting application.

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