Promoter trapping in *Lotus japonicus* reveals novel root and nodule GUS expression domains

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Introduction

T-DNA is widely used in plant genetic screens as a mutagen as well as an enhancer/promoter trap. The random insertion into the genome of a reporter gene under the control of a minimal or no promoter can generate a transcriptional fusion with a nearby gene that is indicative of the endogenous gene expression and/or can cause the interruptions of its function. The two situations may happen concurrently. However, when no obvious mutant phenotype is observed, it can be because: (i) the positioning of the insertion still allows functional transcription; (ii) the transcriptional activation was caused by a cryptic promoter (Foster et al. 1999, Plesch et al. 2000); or (iii) there is functional redundancy. A strictly genetic approach cannot easily overcome this last situation and, in the absence of a mutant phenotype, would provide no functional information about individual gene family members other than pointing to their possible existence. Another situation that cannot be overcome using classical genetics occurs when dealing with genes that perform multiple functions during different developmental stages; hence, pleiotropic phenotypes might sometimes be lethal. Finally, many eukaryotic genes do not have a detectable phenotype when they are mutated, as illustrated by studies in *Saccharomyces cerevisiae* (Ross-Macdonald et al. 1999). In contrast, enhancer/promoter trapping can provide genetic information in the absence of a mutant phenotype.

The development of multicellular organisms involves idiosyncratic gene expression patterns. Reporter gene activations can demonstrate these complex patterns of gene expression that can expand beyond tissues/cells defined as single anatomical units (Geisler et al. 2002); in this paper, we will refer to the entirety of such patterns of a given trapped line as ‘GUS expression domains’ (EDs). The EDs are highly informative and, provided they reflect the expression profile of the endogenous gene, they can be used to assign gene function. This was the case for all biological systems in which extensive EDs collections have been created, such as mouse, *Caenorhabditis elegans*, *Drosophila* (for a review see Bellen 1999) and plants, mostly *Arabidopsis thaliana* (Koncz et al. 1989, Kerbundit et al. 1991, Springer 2000). Aspects of plant development such as embryogenesis (Topping et al. 1994), lateral root formation...
(Malamy and Benfey 1997) and inflorescence development (Campisi et al. 1999) have been dissected using enhancer/promoter trap lines.

An important type of developmental programming in plants, but for which extensive ED collections are missing, leads to the acquisition of an atmospheric nitrogen-fixing symbiosis. This programme is absent in Arabidopsis, and restricted mainly to legumes, which are hence able to grow in nitrogen-poor ecosystems. This confers great agronomical and environmental advantages, and therefore fundamental knowledge of this process is highly desirable.

The legume Lotus japonicus has been introduced as a model plant for genetic nodulation studies (Handberg and Stougard 1992). In recent years, Lotus has been used to positionally clone several key genes involved in the perception of the Rhizobium-derived NOD factors (Radutoiu et al. 2003, Imaizumi-Anraku et al. 2004) and also in the systemic control of nodule number (Krusell et al. 2002, Nishimura et al. 2002). T-DNA tagging was also used to identify a gene for initiation of the bacterial infection thread formation (Schauser et al. 1999) and to link a promoter-less β-glucuronidase (GUS) activation event associated with root epidermis and root hairs to a putative calcium-binding protein (Webb et al. 2000). The utility of these genes will be immense upon their placement onto complex genetic networks required for the orchestration of nodulation pathways.

The formation of the nitrogen-fixing symbiosis requires major cellular specification and programming of plant mechanisms; many aspects of these are still unknown. During early nodulation, a set of developmental pathways, most probably recruited, in part, from lateral root formation (Nutman 1952), utilize non-meristematic but pluripotent pericycle root cells to form nodule primordia (Gresshoff and Delves 1986). Additionally, divisions of cortical cells, which become infected with bacteria, participate in nodule organogenesis (for Lotus, see Szczylowskii et al. 1998). A framework for a Lotus nodule ontogeny has been created based on anatomy alone (Szczylowskii et al. 1998). A more detailed analysis of the different cellular lineages is needed for understanding of nodule formation, similar to the lateral root formation model built on information derived from anatomical studies and promoter-trapped lines in Arabidopsis (Malamy and Benfey 1997).

We have created a collection of L. japonicus GUS-trapped lines related to nodulation and lateral root formation in order to (i) be able to monitor relevant genes that might be lethal when fully mutated; and (ii) identify trap lines that define novel lateral root- and nodule-related EDs. In line CHEETAH, GUS expression foci mark pericycle events linked with both lateral root and nodule primordium (LRP and NP). Line FATA MORGANA is a more specific NP marker that identifies a novel type of cellular event associated with the primordium. In line TIMPA, GUS expression specifies a subclass of cortical events associated with curled root hairs and therefore is likely to participate in NP formation. In line VASCO, GUS expression reveals a saddle-like structure that specifies the nodule vasculature.

Results

Trapping events and T-DNA integration

A total of 281 independent transgenic L. japonicus lines generated through hypocotyl transformation and 540 independ-
Promoter trapping in *Lotus japonicus*

Table 1  Summary of GUS activation in *Lotus japonicus* using two different vectors and the hypocotyl and hairy root system

<table>
<thead>
<tr>
<th>Transformation system</th>
<th>Vector</th>
<th>No. of lines</th>
<th>No. of trapped lines</th>
<th>Percentage GUS-positive lines/roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypocotyl</td>
<td>pAGUSBIN19</td>
<td>233</td>
<td>6 (a)</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>pPCTVgus</td>
<td>48</td>
<td>1 (b)</td>
<td>2.1</td>
</tr>
<tr>
<td>Hairy root</td>
<td>pAGUSBIN19</td>
<td>540</td>
<td>28 (c)</td>
<td>5.2</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>821</td>
<td>33</td>
<td>4.0</td>
</tr>
</tbody>
</table>

\(a\) These include: CHEETAH, FATA MORGANA and VASCO.
\(b\) These include TIMPA.
\(c\) These include BAGEL.

Table 2  Isolation of flanking regions for selected *Lotus japonicus* trapped lines

<table>
<thead>
<tr>
<th>Line</th>
<th>Position relative to T-DNA</th>
<th>Product size (kb)</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHEETAH</td>
<td>Left</td>
<td>2</td>
<td>IPCR</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>0.3</td>
<td>IPCR</td>
</tr>
<tr>
<td>FATA MORGANA</td>
<td>Left</td>
<td>0.8</td>
<td>IPCR</td>
</tr>
<tr>
<td></td>
<td>Right</td>
<td>1.5</td>
<td>TAIL PCR</td>
</tr>
<tr>
<td></td>
<td>Right (a)</td>
<td>0.3</td>
<td>IPCR</td>
</tr>
<tr>
<td>VASCO</td>
<td>Right</td>
<td>0.3</td>
<td>TAIL PCR</td>
</tr>
</tbody>
</table>

TAIL PCR, thermal asymmetric interlaced PCR; IPCR, inverse PCR.

\(a\) The right flanking product isolated for FATA MORGANA using IPCR was included in the TAIL PCR fragment isolated for the same line.

ent hairy root lines were analysed for root- and nodule-specific GUS expression. For the hypocotyl regeneration, it is expected that each regenerated plant represents an independent transformation event since each originated from distinct calli at different ends of one hypocotyl explant. Table 1 summarizes the number of lines obtained with each vector and each transformation system and the number of lines showing GUS expression.

Primary transformants contained one, two or three copies of the T-DNA (Fig. 1A). Single insert T-DNA plants that retained the GUS activation were selected based on Southern blot hybridization of *T₂* progeny from independent plants. For each CHEETAH, FATA MORGANA and VASCO, *uidA* and *nptII* probes located on each side of the T-DNA *HindIII* restriction side were used (shown for CHEETAH with the *uidA* probe, Fig. 1B lanes 5–7; not shown for FATA MORGANA and VASCO). The GUS homzygote lines were selected based on the histochemical GUS test of *T₂* single T-DNA progeny.

The functional transcriporonal GUS fusions in FATA MORGANA (Fig. 1D) and CHEETAH (not shown) were detected on a Northern blot and indicated that the GUS fusions were tissue specific and were stable.

Genomic environment of the T-DNA insertions

To define putative cis-acting elements and possibly associated target genes, T-DNA flanking regions were isolated for three trapped lines. Both left and right border flanking regions were cloned for CHEETAH and FATA MORGANA by inverse PCR (IPCR, Lindsey et al. 1993), and the right one for VASCO by thermal asymmetric interlaced PCR (TAIL PCR, Liu et al. 1995) (Table 2). The comparison between these products and the wild-type sequences amplified across the T-DNA insertion allowed us (i) to establish the re-arrangements that took place in the genomic and transgenic DNA for each insertion; and (ii) to confirm that the isolated left and right flanking sequences belonged to the same genomic locus. We found minor deletions at the T-DNA borders for all three lines and that some of the plant genomic DNA was deleted as well (18 bp for CHEETAH, 7 bp for FATA MORGANA, 14 bp for VASCO). An unexpected T-DNA re-arrangement was found for FATA MORGANA, where 107 bp from the coding sequence of GUS (bp 1,834–1,941 on the pAGUSBIN19 T-DNA) were reinserted immediately after the T-DNA right border. For the three lines described, it is most likely that the T-DNA itself did not undergo major re-arrangements because the Southern blot probed with *uidA* and *nptII* gave signals with a greater size than the estimated minimal one (2.8 and 2.4 kb, respectively) and because the two cassettes had to be functional in order to allow GUS visualization and regeneration on selection medium.

The flanking sequences were then used as a probe to screen a transformation-competent artificial chromosome (TAC) library. For each of FATA MORGANA, CHEETAH and VASCO, a single TAC clone was fully sequenced. We used a sum of pieces of evidence to confirm that the T-DNA for each
line resides on the corresponding TAC sequence, as follows: (i) the Southern blot hybridization patterns with a uidA probe and a flanking genomic DNA probe generated the same size band for a GUS homozygous, single copy T-DNA line (Fig. 1B and C illustrates the CHEETAH example); (ii) IPCR fragments isolated for left and right T-DNA flanking regions are sized by the location of the first restriction site for HindIII and EcoRI, respectively; upon sequencing the TAC clones, these locations were confirmed on both sides of the T-DNA; (iii) Southern blotting on genomic DNA isolated from T-DNA lines with uidA and nptII probes (each residing on the 2.4 and 2.8 kb side of HindIII of pAGUSBIN19 T-DNA, respectively) generated fragments of the predicted size based on their corresponding TAC; and (iv) IPCR fragments isolated independently for each left and right T-DNA flanking region were used to design a wild-type genomic PCR across each T-DNA insertion site; these PCR’s always generated single products for the three lines described.

The FATA MORGANA TAC clone of 93 kb, LjT22D23 (GenBank accession no. AP008242) contained mainly regions of repetitive and retrotransposon-like DNA, with only one known gene, LjENOD40–2, situated about 4 kb upstream of the insertion and orientated opposite the GUS gene on the T-DNA (Fig. 2A). Out of the three TAC clones described in this study, the FATA MORGANA clone had the lowest gene density (see below).

For CHEETAH, two TAC libraries (Men et al. 2001, Sato et al. 2001) constructed in genotype Gifu and Miyakojima, respectively, were screened. The Gifu sequence of about 8 kb (4 kb on each side of the insertion) matched the Miyakojima one. The CHEETAH TAC clone of about 86 kb, LjT39G20 a and b (deposited in GenBank under accession nos AP008240 and AP008241), revealed the integration of the T-DNA in a gene-rich region (see Fig. 2B). In the left flanking region, three different putative proteins spanning 8.3 kb showed no homology to proteins of known function. The closest open reading frame was located 3.6 kb upstream from the left T-DNA border. In 40 kb of the right flanking region, putative genes were predicted with diverse functions including two genes containing EH domains (Wong et al. 1995). The EH-encoding genes have a protein–protein interaction surface with an asparagine–proline–phenylalanine motif as an optimal ligand. Preliminary data indicate that the Lotus eh genes are transcribed and are part of a gene family with at least three members (unpublished data). For the VASCO TAC clone of 49 kb, LjT07P16 (GenBank accession no. AP008243) (Fig. 2C), the T-DNA insertion is positioned at 23 kb, just 0.8 kb upstream of an AAA ATPase-encoded domain (Patel and Latterich 1998). This domain is common to all ATPases and is defined by a conserved 230–250 amino acid sequence that includes the Walker signature sequences of P-loop ATPases and other regions of similarity unique to AAA proteins. This VASCO flanking region has highest homology to a predicted rice spastin-like gene and a rice transitional endoplasmic reticulum ATPase-like protein. Further downstream, a full-length eukaryotic translation initiation factor-5A can be deduced based on homology with a Brassica napus accession. On the other side of the insertion, a putative Arabidopsis thaliana cdc2 protein kinase homologue can be predicted to encode seven exons spanning >8 kb of genomic sequence.

**Trapping spectrum**

Histochemical GUS assays were used to detect the different cellular types expressing the reporter gene at different time points for each independent line. Several lines, including FATA MORGANA, TIMPA, HYENA, DONUT and BAGEL, were isolated that had *Mesorhizobium loti*-dependent GUS expression patterns. Although for these lines it is mostly the symbiotic (nodule primordia, nodules and nodule vasculature) and/or potentially symbiotic tissue (pericycle) that showed the activation, connective tissue of the anther of FATA MORGANA also showed GUS expression. The other class of lines had inoculant-independent GUS expression (in LRP and root tip), including CHEETAH but, when nodulated, also expressed GUS in symbiotic tissue. The expression of all lines is summarized in Table 3, and a more detailed description of the GUS expression of four of the lines is given below.
Table 3  Nodule and root GUS expression spectrum for selected *Lotus japonicus* trapped lines  

<table>
<thead>
<tr>
<th>Line</th>
<th>Root GUS expression domains</th>
<th>Nodule GUS expression domains</th>
<th>Other GUS expression domains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rt</td>
<td>lrp</td>
<td>lrv</td>
</tr>
<tr>
<td>CHEETAH</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>FATA MORGANA</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TIMPA</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VASCO</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DONUT</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BAGEL</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HYENA</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

rt, root tip; lrp, lateral root primordium; lrv, lateral root vasculature; ccd, cortical cell divisions; p, pericycle; np, nodule primordium; nv, nodule vasculature; noc, nodule outer cortex; nic, nodule inner cortex; ui, uninfected zone of the infected area of the nodule.

a Connective/vascular tissue of the anther; ND, not determined.
b Root area around nodule.

The *FATA MORGANA* expression domain

The FATA MORGANA ED has the following sub-domains: (i) certain root pericycle cells at the initial stages, 3 d after inoculation with *M. loti* (Fig. 3A); (ii) the core cells of the nodule primordium and two arrays of cells each flanking the primordium on one side (arrows) (Fig. 3B–E); (iii) nodule cells including inner cortex, possibly the boundary cell layer, uninfected cells of the infected zone and certain cells associated with the vascular bundles of the nodule (arrowheads) (Fig. 3F–H); (iv) vascular/connective tissue of the anther only when the flower is closed and about 2 mm length (Fig. 3I, J). The pericycle cell layer has very low GUS expression, which is barely detectable in whole mount roots. This expression pattern is transient as it disappears at the time of nodule primordium formation. However, later during nodule development other pericycle cells, flanking the xylem pole adjacent to an emerging nodule and running along its length, express GUS (Fig. 3B, C). These arrays of cells always accompany the emerging nodule, as illustrated by the fact that when two nodules emerge at the same site, each one is flanked by an array (Fig. 3D and close up of the indicated area in E).

The CHEETAH expression domain

An overnight water imbibition of CHEETAH seed induced strong GUS expression in the radicle tip (the meristematic as well as the columella cells) that persists throughout the root development (Fig. 4A). A multitude of early and late stages of lateral root and nodule ontogeny show GUS activation in CHEETAH (Fig. 4B). Malamy and Benfey (1997) have previously described eight lateral root ontogeny stages in *A. thaliana*, using a combination of a marker line collection and anatomy. Fig. 4C illustrates the CHEETAH expression at the point which is equivalent to stage III in *Arabidopsis* LRP (primordium is composed of an inner layer and an outer layer that has just undergone one cellular division to generate two outer layers). Fig. 4E illustrates CHEETAH GUS staining during stage Vla. Other CHEETAH GUS expression sub-domains are related to nodule ontogeny: (i) division foci in cortical cells rather than pericycle cells (compare Fig. 4D with C); and (ii) later stages of nodule ontogeny (Fig. 4F). A common feature for the lateral root and nodule GUS expression is the activation in vascular bundles: the base of both organs transiently expressed GUS (Fig. 4G, H).

The TIMPA GUS expression domain

Upon inoculation with *M. loti*, GUS expression was seen in the dividing cells of the inner cortex that are in the vicinity of a curled root hair (Fig. 5A). Not all the root hairs that curled had associated GUS expression. A characteristic TIMPA GUS expression subdomain is represented by cortical division foci (Fig. 5B). In our whole-mount specimens, TIMPA NP GUS staining is different from that of FATA MORGANA, as the latter stained core primordia cells only, whereas TIMPA GUS was visualized in more cells of nodule primordia cells (compare Fig. 3B, 5C). Also the TIMPA NP was not surrounded by the GUS-expressing cells as was the case for CHEETAH (compare Fig. 5C, 4F). In the fully differentiated, nitrogen-fixing nodule, the expression remained restricted to the nodule outer cortex (Fig. 5D).

The VASCO GUS expression domain

The GUS expression in VASCO is very specific, restricted to the vascular bundle of the nodule (Fig. 5E, F). Although the vasculature of the nodule is connected to that of the root, the whole-mount specimens did not show any GUS expression in the root. In order to determine the pattern of vascular differentiation within the nodule, the nodule interior was removed (Fig. 5G). A saddle-like shape, spanning the root stele with four


individual nodule vascular strands originating at the corners, was found to express GUS. All nodule vascular strands expressed GUS regardless of their number in a nodule (data not shown).

**FATA MORGANA nodule expression can be reiterated by the upstream 2 kb sequence**

A set of six fusion constructs (three for each orientation) were designed to test if a bi-directional regulatory element lies between the transcriptional start of LjENOD40–2 and GUS because (i) LjENOD40–2 (Flemetakis et al. 2000) and FATA

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**Fig. 3** FATA MORGANA ED. GUS expression was found in: (A) pericycle (p) at 3 d post-inoculation; (B–E) nodule primordium and two arrays of cells (arrows); (E) represents a close up of the area indicated in (D); (F) nodules. (G and H) GUS expression is restricted to the inner cortex, possibly including the boundary cell layer, the uninfected cells of the infected area and certain cells associated with the vascular bundle of the nodule (arrowheads). (I) Closed flower, 2 mm length. (J) Amber connective/vascular tissue.

**Fig. 4** CHEETAH ED. GUS expression was found in: (A) root tip; (B) different lateral root and nodule stages. (C) Staining of the equivalent of stage III in Arabidopsis lateral root development. IL, inner layer; OL1, outer layer 1; OL2, outer layer 2. (D) Cortical cell division foci. (E) Staining of the equivalent of stage VIa in Arabidopsis lateral root development. (F) Young nodule. (G) Lateral root. (H) Nodule.

MORGANA EDs are similar; (ii) the physical proximity of the two genes; and (iii) their opposite orientation (Fig. 6A). These constructs were amplified from FATA MORGANA and contain plant DNA as well as 684 bp of GUS upstream transgenic vector sequence (pAGUSBIN19; Lindsey et al. 1993). This region is M13 derived and does not contain any obvious sequences that may act as TATA boxes. Agrobacterium rhizogenes was used as a delivery system for the fusion constructs, and the resulting transgenic hairy roots were analysed for GUS expression following inoculation with M. loti. One nodulation stage (4 weeks post-inoculation) was tested for GUS activity and, therefore, in these initial experiments, the early pericycle was not assayed. The promoterless pCAMBIA1391Z binary vector (CAMBIA, ACT, Australia) used for all the constructs sometimes resulted in GUS expression in the root vasculature,
plant. The other three constructs never generated any detectable GUS activity in the whole-mount roots under our experimental conditions.

Construct 1F contained the 2 kb sequence immediately in front of the GUS ATG start codon and its nodule expression matches that of FATA MORGANA (Fig. 6B, C) with different intensities in different transgenics (Fig. 6D, E). Construct 2R represents the putative LjENOD40–2 promoter, and was found to express GUS in the vasculature of all nodules (Fig. 6G) and in some root vasculature (Fig. 6F). We observed that the root vasculature faded and eventually disappeared acropetal from the last nodule on both the main and the lateral roots (Fig. 6F, arrowhead). Whenever a lateral root did not contain a nodule, root vasculature GUS expression was absent (Fig. 6F, G, arrows). Construct 3F retained the inner nodule expression seen for construct 1F in some nodules of different transgenics and occasionally expressed GUS in the nodule vasculature (Fig. 6H, I).

**Discussion**

We have created a collection of trapped lines in *L. japonicus* that have GUS patterns which mark events during lateral root and nodule development both spatially and temporally. Novel EDs that group more than one type of GUS expression were identified. These are believed to originate from single genetic loci because: (i) single insert T-DNA lines homozygous for GUS were used for all lines described; (ii) GUS activations were stable and constant in different generations, and in a large number of individual plants, segregation of different GUS expression subdomains was never observed. The precise matching between these domains and the expression of an endogenous gene, together with establishing if they are caused by a genuine or cryptic promoter elements, requires further detailed examinations.

Apart from the ultimate definition of endogenous gene function, and independent of it, the trapped lines have been used as cellular markers for ontogeny models (Malamy and Benfey 1997), for specific cellular marking/selection (Birnbaum et al. 2003) or for ectopic gene expression/mutant background analy-

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**Table 4**  Number of hairy roots generated with the six different GUS fusion constructs

<table>
<thead>
<tr>
<th>Construct</th>
<th>Experiment I</th>
<th></th>
<th>Experiment II</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of hairy roots</td>
<td>No. of GUS+</td>
<td>No. of hairy roots</td>
<td>No. of GUS+</td>
</tr>
<tr>
<td>pCambia1305 (35S::GUS)</td>
<td>–</td>
<td>–</td>
<td>49</td>
<td>17</td>
</tr>
<tr>
<td>pCambia1319Z (AGUS)</td>
<td>–</td>
<td>–</td>
<td>39</td>
<td>10</td>
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<tr>
<td>1F</td>
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</table>
sis. Nodule organogenesis involves the specification of different cellular types, some of which share similarities with other organs, such as lateral roots, and others which are specific to nodulation. The GUS expression found at the onset of nodulation in FATA MORGANA, CHEETAH and TIMPA offers opportunities to describe the ontogeny of different cellular types participating in the organization of meristematic centres of nodules and lateral roots. Specific FATA MORGANA and TIMPA GUS expression subdomains are associated with nodule ontogeny which relates to pericycle and cortex differentiation, respectively, while some CHEETAH GUS expression subdomains mark both pericycle and cortical initiation of both lateral roots and nodules. Various GUS expression subdomains relate to the later nodule development stages, in a non-overlapping manner: infected cells of the nodule interior and possibly the boundary cell layer are marked in the FATA MORGANA line, nodule outer cortex is marked in the TIMPA line and different cells of the nodule vasculature are marked by GUS expression subdomains of FATA MORGANA, CHEETAH and VASCO. Therefore, the trapping events present in this collection cover a wide spectrum of GUS expression and represent excellent tools for legume nodule and lateral root ontogeny models.

The association of different GUS expression subdomains within one line that we found in FATA MORGANA was unpredictable. Two types of specifications may co-exist: one that relates to pericycle and the site of nodule initiation and the other one that relates to cells inside the infected area of the nodule. Early nodulin40 legume family members, one of which is encoded 4 kb upstream of the FATA MORGANA insertion, LjENOD40–2, have been shown to be expressed in a similar manner (Kouchi and Hata 1993, Flemetakis et al. 2000, Gronlund et al. 2005). Also, the fact that pericycle cells adjacent to phloem express GUS in a temporally regulated manner that relates to the initiation and the emergence of the nodule could be associated with the long postulated ‘stele factor’ (Libbenga and Harkes 1973) known to affect the radial distribution of nodules.

In order to clarify the possible genetic links, we attempted to define the regulatory DNA regions that dictate the FATA MORGANA GUS expression. We utilized GUS fusions with DNA sequences from the upstream vicinity of the T-DNA insertion. Agrobacterium rhizogenes-generated composite plants were previously shown to mimic the A. tumefaciens stable transgenics in regard to transgene expression (Martirani et al. 1999), so we utilized transgenic hairy roots to identify the FATA MORGANA regulatory elements. Constructs 1F and 3F incorporated 2 and 4 kb of plant upstream DNA with GUS fusions in the correct orientation for pABIN19GUS. They were able to reproduce the FATA MORGANA nodule GUS expression. This indicates that these sequences contain at least part of the elements required for the FATA MORGANA ED in transformed hairy roots. It is unlikely that a combination of endogenous sequences and the transgenic ones (light blue box, Fig.
6A) is responsible for the GUS activation, because: (i) the size of the GUS fusion transcript (Fig. 1D) is indicative of the usage of an unspecified TATA box from the endogenous left flanking region, situated at least 0.3 kb (assuming no splicing event in the fusion transcript) upstream of the insertion; and (ii) no minimal TATA boxes are present in this region. Constructs 1R and 3R represent the reverse orientation of 1F and 3F, respectively, and they failed to give detectable GUS expression in whole-mount nodulated roots in two independent experiments. This can indicate that (i) the endogenous sequences responsible for the expression of construct 1F and 3F do not function in both orientations; or (ii) the putative endogenous sequence that could work in both orientations is blocked by other transcriptionally active sequences in this area that normally require more genomic context than the sequence used in our fusions. Our recent data indicate that the last situation is likely.

Constructs 2F and 2R represent the 2 kb of sequence upstream of the LjENOD40–2 gene in the correct and inverse orientation, respectively. Previous GUS promoter fusion studies in other legumes (Fang and Hirsch 1998, Martirani et al. 1999) attributed the activity of different ENOD40 genes to its approximately 2 kb upstream sequence. In situ hybridizations indicated that LjENOD40–2 has strong expression in the nodule vascular bundles, and reverse transcription–PCR studies showed that other organs, including roots, express the gene (Flemetakis et al. 2000). We found that construct 2R strongly expressed GUS in nodule vasculature and that the root vasculature GUS expression had a special distribution: it faded away in the main root after the last nodule position and it was present in the vasculature of lateral roots only if a nodule developed on that lateral root and also faded away after the last nodule. To our knowledge, this pattern was not described before for any ENOD40 gene. The two L. japonicus ENOD40 genes have slightly different expression patterns (Flemetakis et al. 2000), which can reflect distinctive functions (Takeda et al. 2005). A transport function was proposed for the soybean ENOD40 genes (Kouchi and Hata 1993) and our LjENOD40–2::GUS fusion (construct 2R) may also support this possibility. Construct 2F did not give any GUS expression, suggesting the absence of a bi-directional element that could function with no other combinatorial elements. In summary, our analysis of the promoter fusion constructs indicates a complex transcriptional regulation at the FATA MORGANA locus. It is possible that elements that regulate LjENOD40–2 also regulate FATA MORGANA GUS. These elements could also act on another transcriptional unit situated in the vicinity of this locus.

Our promoter trapping programme in L. japonicus has resulted in the generation of a collection of lines that express the GUS reporter gene in a stable and developmentally regulated manner. More thorough studies on the molecular mechanisms that define the novel expression domains of the lines described here in combination with anatomical knowledge of nodule ontogeny will enlighten our understanding of developmental and genetic programming of nodule formation.

Materials and Methods

Bacterial strains, and vectors for plant transformation

Agrobacterium tumefaciens strains GV3101 and LBA4404, and A. rhizogenes strain AR10 in combination with different binary vectors were used. Agrobacterium strains were grown in YEB medium (5 g of beef extract, 1 g of yeast extract, 5 g of peptone and 5 g of sucrose 1–1 of medium, 1 mM MgCl₂; pH 7.0; 1.4% Bacto-agar for solid medium) with antibiotics in appropriate concentrations at 28.0°C. Mesorhizobium loti strain NZP2235 was grown in YMB medium (2 g of mannitol, 0.4 g of yeast extract, 0.5 g of K₂HPO₄, 0.2 g of MgSO₄·7H₂O, 0.1 g of NaCl 1–1 of medium, pH 6.8; 1.5% Bacto-agar in solid medium) at 28.0°C and was used to inoculate L. japonicus ecotype Gifu B-129–56 for the induction of nodules.

Two different binary vectors with a promoter-less GUS cassette within the T-DNA border repeats were used for plant transformation. The vector pBIGUSBIN19 had a promoter-less GUS gene at the left T-DNA border (Lindsey et al. 1993). The other vector, pCPTV/GUS (Koncz et al. 1994), had a promoter-less GUS construct at the right T-DNA border.

Plant transformation and growth, nodulation assay

The hairy root and hypocotyl transformation was done on seedlings of L. japonicus ecotype Gifu B129 as described by Stilller et al. (1997). Plantlets with 1–2 cm long roots (hairy roots and no transformed roots) were transferred to 1/2× B5 agar slopes with 1/2× B5 vitamins. Two to 4 d later, these plants were transferred to 1/2× B&D slopes (Broughton and Dillow 1971) supplied with 2 mM (NH₄)₂ as (NH₄)₂SO₄ and 1 mM KNO₃. Bacto-agar 1.4% and pH 6.8 was used. Diluted (1 : 1 with YMB) 3-day-old culture of M. loti NZP2235 was applied to growing root tips using a micro-pipette.

Plants were grown in the greenhouse conditions in a horticultural mix (Fafard Mix NO. 4 from Conrad Fafard, Inc., Agawam, MA, USA) and watered sparingly with Miracle Gro (Miracle Gro 15–30–15, from Scotts Miracle-Gro Products, Inc., Port Washington, NY, USA) and water. Pods were harvested at the ripening stage (brown pod colour); seeds were isolated and kept in a cool room for vernalization. Seedlings were scarified by rubbing them against sand paper (aluminium oxide sand paper, 150 grit, fine, from Ace Hardware Corp., Oak Brook, IL, USA) before sterilization in 3.0% H₂O₂ as described previously. For some of the nodulation tests, plants were grown in vermiculite and watered with B&D solutions supplied with 1 mM KNO₃.

Histochemical GUS assay and sectioning

Roots inoculated with M. loti strain NZP2235 were excised after different intervals of incubation and put into GUS assay buffer as described by Jefferson et al. (1987), and incubated overnight at 37°C. A second type of GUS buffer (Kosugi et al. 1990) was used for confirming the FATA MORGANA flower GUS expression. The stained tissues were observed under a stereomicroscope (Olympus SZH) and pictures were taken.

For sectioning (Fig. 3A,G,H), nodulated roots explants were first GUS stained then fixed overnight in 70% FAA, then dehydrated through an ethanol series starting with 20–100% for 30 min each. After overnight incubation in 100% ethanol, nodulated root segments were incubated in a series of increasing xylenol concentration in ethanol, up to 100% xylenol. In the same way, the xylene was replaced with histowax, up to 100% at 60°C, then specimens were vacuum infiltrated for 3 min and allowed to infiltrate for several days. A Rotary microtome was used to make 10–15 μm thin sections. Hand sectioning (100 μm thin) (Fig. 3C, D, E) was done as described by Larkin et al. (1996).
Southern and Northern blotting, PCR andTAIL PCR. TAC isolation and sequencing

Plant genomic DNA was isolated as described (Dellaporta et al. 1983). Genomic Southern blotting was carried out after digesting the genomic DNA either by EcoRI (in the case of lines transformed with pPCTVgUS) or by HindIII (in the case of lines transformed with the vector pAGUSBVIN19). RNA was isolated using the hot phenol method (deVries et al. 1982) and 20 μg were loaded for each sample. The RNA was transferred to a Hybond membrane which was stained in 0.03% methylen blue in 0.3 M sodium acetate at pH 5.2 for 45 s, then de-stained in sterile water for 30 min at 65°C. A 1,887-bp fragment with the GUS coding region was used as the probe for both Southern and Northern hybridizations.

IPCR and TAIL PCR were used to amplify flanking regions for CHEETAH, FATA MORGANA and VASCO. IPCR was done essentially as described by Lindsey et al. (1993), except that 6 μg of genomic DNA was used for digestion, and 100 ng of ligated DNA was used as the template in a PCR mix as described by Gibco-BRL for eLONgase mix.

TAIL PCR was done essentially as described by Liu et al. (1995). A 10 ng aliquot of VASCO genomic DNA was used in the primary PCR using the forward specific primer, 5′CTCTGTAGACCGGTTTCCCAACC3′ and the reverse degenerate 5′(A/T)GTGNGA(A/T)ANCANAGA3′. Amplified products that had the right shift were cloned into pGEM T-easy vector and sequenced.

Plasmid construction for promoter region analysis

PCR was performed using the Expand Long Polymerase kit (Roche) and FATA MORGANA genomic DNA as a template. Four primers with the Ncol restriction site incorporated in their sequence were used in three different combinations as follows: for constructs 3F and 3R, 5′GACCATGTGGAAATTITTTAAAGAAC3′ and 5′GCCCGTGGGTITCAACCTGGGTAC3′ for constructs 2F and 2R, 5′GACCATGGGAAAATTITTTAAGAAAC3′ and 5′CTCCATGGTTTCGTTAGGTTGGACTC3′; and for constructs 1F and 1R, 5′CGGCATGGCACTGCAACTCTACGAAA3′ and 5′GCCATGGCTGTTTATACACGTTGGACTG3′. DNA manipulations were carried out using standard protocols (Sambrook et al. 1989). Junction sites were confirmed by sequencing.

TAC screening and sequencing

A genomic library of L. japonicus accession ‘ Miyakojima’ MG-20 (Sato et al. 2001) was screened using the three-dimensional pooling method with oligonucleotides synthesized to amplify the flanking region sequenced for CHEETAH, FATA MORGANA and VASCO. The corresponding TAC clones isolated were sequenced using the bridging shotgun method.

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