Mesorhizobium loti* Increases Root-Specific Expression of a Calcium-Binding Protein Homologue Identified by Promoter Tagging in *Lotus japonicus

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A promoter tagging program in the legume *Lotus japonicus* was initiated to identify plant genes involved in the nitrogen-fixing symbiosis between legumes and rhizobia. Seven transformed plant lines expressing the promoterless reporter gene *uidA* (β -glucuronidase; GUS) specifically in roots and/or nodules were identified. Four of these expressed GUS in the roots only after inoculation with nodule-forming *Mesorhizobium loti*. In one line (T90), GUS activity was found in the root epidermis, including root hairs. During seedling growth, GUS expression gradually became focused in developing nodules and disappeared from root tissue. No GUS activity was detected when a non-nodulating mutant of *M. loti* was used to inoculate the plants. The T-DNA insertion in this plant line was located 1.3 kb upstream of a putative coding sequence with strong homology to calcium-binding proteins. Four motifs were identified, which were very similar to the “EF hands” in calmodulin-related proteins, each binding one Ca^{2+} . We have named the gene *LjCbp1* (calcium-binding protein). Northern (RNA) analyses showed that this gene is expressed specifically in roots of *L. japonicus*. Expression was reduced in roots inoculated with non-nodulating *M. loti* mutants and in progeny homozygous for the T-DNA insertion, suggesting a link between the T-DNA insertion and this gene.

Additional keywords: *Agrobacterium tumefaciens*.

Symbiotic nitrogen fixation in legumes is a result of a complex interaction between the plant and symbiotic soil bacteria known as rhizobia. This process has been the subject of intense study in recent years. While the molecular and genetic basis of signal exchange has been elucidated in rhizobia (Long, 1996; Mylona et al. 1995; Schultze and Kondorosi 1998), progress in the isolation of corresponding plant genes has been hampered by the lack of appropriate technology. Many plant genes, specifically expressed in nodules or in

roots in response to Nod factors produced by rhizobia, have been identified by differential screening of cDNA from roots and nodules (Kapranov et al. 1997; Gamas et al. 1996, reviewed by Mylona et al. 1995; Schultze and Kondorosi 1998). The limitation of this method is that important genes expressed transiently, and/or at very low levels, may not be detected. Chemical and radiation mutagenesis programs have been very successful in generating nodulation and fixation mutants in legumes, but isolation of the mutated genes is more difficult (Redhead and Palme 1996).

The development of transposon and T-DNA tagging techniques in plants has opened up new possibilities for identifying genes involved in nodulation and nitrogen fixation. These approaches have been used successfully in model plants such as *Arabidopsis* and tobacco (Feldmann 1991; Fobert et al. 1991; Koncz et al. 1990; Teeri et al. 1986; Topping et al. 1991), and in certain crop plants such as potato (Lindsey et al. 1993), but none of these species are able to form symbiotic interactions with rhizobia. However, *Medicago truncatula* and *Lotus japonicus* have recently been identified as useful model legumes, because of their small genomes, their autogamy, and the relative ease with which they can be transformed (Barker et al. 1990; Cook et al. 1997; Handberg and Stougaard 1992; Jiang and Gresshoff 1997). A transposon tagging program in *L. japonicus* with the maize *Ac* transposon (Thyckjær et al. 1995) has recently resulted in the identification of a plant regulatory gene required for infection thread and nodule primordium development (Schauser et al. 1999). Chemical mutagenesis programs have also been developed in this species (Szczyglowski et al. 1998). The development of a protocol for *Agrobacterium rhizogenes*-mediated transformation and regeneration of *L. japonicus* (Stiller et al. 1997) has led to the initiation of a T-DNA tagging program with this system (Martirani et al. 1999). The co-transformation of *rol* genes in *A. rhizogenes*-mediated transformations is a complicating factor, because expression of *rol* genes disturbs the hormone balance of the plant. This has the potential to affect expression of nodule-specific genes, although three well-characterized nodulin promoters did not appear to have changed their expression pattern (Martirani et al. 1999).

Transformation by *Agrobacterium tumefaciens* with a binary vector avoids this potential problem. Here we describe the first results of a T-DNA tagging program with *A. tumefaciens* in *L. japonicus*. We have used a promoter-trapping gene

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construct to identify transformants with T-DNA insertions within or close to genes specifically expressed in nodules or in response to rhizobia. The construct contains the promoterless glucuronidase *uidA* gene (β -glucuronidase; GUS) adjacent to the left border of the T-DNA (Topping et al. 1991), and has been used to identify and characterize tagged genes in *Arabidopsis thaliana* (Lindsey and Topping 1996; Wei et al. 1997). Here, we describe the identification of seven tagged lines with GUS activity in root nodules of *L. japonicus*, in some of which GUS expression is induced only after inoculation with *M. loti*. One of the tagged lines is characterized in more detail, and shown to have the T-DNA inserted adjacent to a gene with strong homology to genes encoding calcium-binding proteins.

RESULTS

Analysis of GUS expression in tagged lines.

A total of 284 kanamycin-resistant transformants of *L. japonicus* were produced by *A. tumefaciens*-mediated transformation with the binary vector pAgusBin19. The presence of the two transgenes (*uidA* and *nptII*) was investigated by polymerase chain reaction (PCR) in 155 of these plants. Of those, 74% (115) were positive for both transgenes. In the remaining 26% either the *nptII* gene (21%) or the *uidA* gene (4%) or neither (1%) was amplified. The next step was to perform a histochemical analysis for GUS activity in the transformants. While 97% (275) did not express GUS activity consistently in any tissue, the remaining 3% (9) expressed reproducible GUS activity in roots and/or nodules. These GUS-positive transformants (T90A, T90B, T554, T563, T567, T655A, T655B, T660, and T711) were analyzed in more detail. The results are summarized in Table 1. The transformants from T90 and T655, which were labeled A and B, were regenerated from the same transformed callus culture. Transformants T90A and T90B had identical GUS expression patterns, as did T655A and T655B. Therefore, plants A and B were considered duplicates of the original transformation event, reducing the number of independent transformed plants to 200.

The remaining seven independent primary transformants had different GUS expression patterns, but could be classified into two distinct groups. Three lines (T554, T655, and T660) expressed GUS in nodules and other root tissue both before and after inoculation with *M. loti*, and four plants (T90, T563, T567, and T711) expressed GUS in root and nodule tissue only after inoculation with *M. loti* (Table 1). In the transformants T655 and T660, GUS expression was observed consistently in root tissue as well as in nodules. In T655, GUS activity was confined to the root vascular tissue (Fig. 1A), while in the latter, GUS activity was evident in all root tissue except the root tip (Fig. 1B) and the most basal part of the root. GUS activity was also observed in other tissue in some cases, notably in connective tissues of anthers of immature, mature, and senescing flowers in T655 (Fig. 1G, Table 1), and in roots of germinating embryos in transformant T554 (Fig. 1H).

In the four lines where GUS expression was detected in roots and nodules only after inoculation with *M. loti*, T567 and T711 had the GUS activity confined to nodules. In T567 GUS activity was found in immature and mature nodules, while in T711 it was detected in mature and senescent nodules. In contrast, both T90 and T563 were GUS positive at

every stage of nodule development. However, in T90, GUS activity was strongest in developing and young nodules with diminishing activity in older nodules (Fig. 1E and F). In the root system of T90, post-inoculation GUS activity was present in the epidermis (Fig. 1C), and interestingly was observed in the root hairs of root hair cells (Fig. 1D). As nodule development progressed, GUS activity decreased in the root system, and gradually became confined to the emerging nodules.

Preliminary genetic and molecular analysis of GUS-expressing tagged lines.

The presence of the *uidA* and *nptII* genes in the seven GUS-positive lines was confirmed by PCR, and the number of T-DNA insertions in each line was determined by Southern blot hybridization, such as the one shown in Figure 2A. A map of the T-DNA region of pAgusBin19, indicating the internal *EcoRI* and *HindIII* restriction sites, is shown in Figure 2B. The result confirmed that both T90A and B lines had only one T-DNA copy of the same size, whereas the other six lines had two or three copies (Fig. 2A, Table 2). The inheritance of GUS expression and T-DNA was investigated in the T₂ progeny. Progeny from all except one of the T₁ plants expressed GUS in the seedling root nodules (Table 2). In T567, no GUS expression was found in any of the 41 progeny (28 embryos and 13 seedlings). In this line, a *HindIII* fragment of approximately 1.1 kb hybridized to the *uidA* probe (Fig. 2). The expected minimum size of a hybridizing *HindIII* fragment is 2.8 kb (see Figure 2B), suggesting that some rearrangement of the T-DNA had taken place in this line. Some GUS activity was detected in germinating embryos of four lines (Table 2), three of which expressed GUS in nodules and one (T655) in both roots and nodules. The inheritance and segregation of the GUS expression phenotype in the T₂ progeny was investigated, and the results are shown in Table 2. In four lines (T563, T655, T660, and T711) the percentage of seedlings with GUS expression in nodules is nearer a 2:1 ratio than to the expected 3:1 ratio (assuming that only one T-DNA copy is responsible for the observed GUS phenotype). In seedlings of T90, GUS activity was found only in roots and nodules. This line contained only one T-DNA copy, and the 72% of seedlings expressing GUS suggested a 3:1 ratio ($\chi^2 = 1.44$), as op-

Table 1. β -Glucuronidase (GUS) localization in primary transformants of *Lotus japonicus*, containing a promoterless *uidA* gene

Plant no.	Plant tissues							Seeds, seed pods
	Root nodules	Roots ^a		Stems	Leaves	Shoot		
		Before inoc.	After inoc.			tips	Flowers	
Control	— ^b	—	—	—	—	—	+/-	—
T554	+ ^c	+/- ^d	+/-	(+) ^e	—	—	—	—
T655	+	+	+	(+)	—	—	+	+/-
T660	+	+	+	—	—	—	+/-	—
T90	+	-	+	(+)	—	—	+/-	—
T563	+	—	+/-	—	—	—	+/-	—
T567	+	—	—	(+)	—	—	+/-	ND ^f
T711	+	—	—	—	—	—	+/-	—

^a Roots not including nodules.

^b No GUS activity.

^c GUS activity found in more than 50% of samples.

^d GUS activity found in less than 50% of samples.

^e GUS activity may be due to fungal contamination.

^f Not determined.

posed to a 2:1 ratio ($\chi^2 = 2.78$). All progeny expressing GUS also contained the *uidA* gene (determined by PCR).

GUS expression phenotype in T90 is not induced by non-nodulating *M. loti*, and is linked to the T-DNA insertion.

T90 was characterized further for two reasons. First, GUS activity was induced only after inoculation with the *M. loti*

wild-type strain NZP2235, with GUS expression localized in root hairs, some of which curled in response to *M. loti* (Fig. 1D). Second, the primary transformant contained only a single copy of T-DNA, simplifying isolation of plant DNA adjacent to the insertion.

We compared the GUS expression patterns of T90 inoculated with the wild-type *M. loti* strain NZP2037 and a non-nodulating mutant, PN4047, derived from NZP2037. This

Fig. 1. Histochemical localization of β -glucuronidase (GUS) activity in *Lotus japonicus*. **A**, GUS activity in root vascular tissues and mature root nodule from primary transformant T655. **B**, GUS activity in root tissue except the root tip of T660, prior to inoculation with *Mesorhizobium loti*. GUS expression in primary transformant T90 after inoculation with *M. loti* in **(C)** root epidermal cells, **(D)** root hair cells, **(E)** immature and mature nodules, and **(F)** mature and senescent nodules. **G**, GUS activity in anther connective tissue in primary transformant T655. **H**, GUS activity in root of 7-day-old stunted, germinating embryo (T_2) of transformant T554. Magnification bar = 0.8 mm, except for **D**, where bar = 0.3 mm.

mutant has a Tn5 transposon inserted in the *nodC* gene (Chua et al. 1985; Scott et al. 1996), and is therefore unable to make a Nod factor in response to root exudates. Seedlings of T90 inoculated by NZP2037 displayed GUS activity as described above, with GUS staining evident in the roots after inoculation and gradually becoming confined to the nodules as they developed (Fig. 3A,C,E, and F). In contrast, the seedlings inoculated by the NodC mutant did not show any evidence of GUS expression in the roots after inoculation (Fig. 3B and D).

Cloning and analysis of *L. japonicus* DNA adjacent to T-DNA in T90 line.

The 3.8-kb *Hind*III fragment from T90 hybridizing with the *uidA* probe suggested that approximately 1 kb of it was *L. ja-*

ponicus DNA upstream of the T-DNA. The remaining 2.8 kb consisted of T-DNA from the left border to the unique *Hind*III site at the 3' end of the *uidA* gene (Lindsey et al. 1993; Topping et al. 1991). Cloning of the 1-kb *L. japonicus* DNA was achieved by inverse PCR as described by Lindsey et al. (1993), with primers from the *uidA* sequence. Nucleotide sequencing of the left T-DNA border and adjacent *L. japonicus* DNA revealed that the first 95 bases of the T-DNA left border were absent (data not shown), presumably as a result of the T-DNA insertion. This clone was then used as a probe in a Southern hybridization experiment with DNA from T₂ prog-

Fig. 2. **A**, Southern hybridization of *Lotus japonicus* wild-type plants (C142) and five primary transformants. Genomic DNA cut with *Hind*III + *Eco*RI (HE) or *Hind*III (H) was hybridized to *uidA* gene probe. Control plant C142 was regenerated but not transformed. **B**, Map of T-DNA region of pΔgusBin19 indicating *uidA* and *nptII* genes, left (LB) and right (RB) borders, and restriction sites for *Eco*RI (E) and *Hind*III (H).

Table 2. Number of T-DNA copies in primary transformants (T₁), percent germination of T₂ seedlings, and percent T₂ seedlings with β-glucuronidase (GUS) activity in embryos and root nodules

Plant no.	T-DNA copy no.	Percent germination	Percent GUS expression	
			Embryos	Nodules
Control	0	83 (79/95)	0 (0/20)	0 (0/102)
T90	1	73 (146/200)	0 (0/12)	72 (192/268)
T554	2	55 (47/85)	42 (5/12)	81 (25/31)
T563	2	78 (70/90)	47 (9/19)	62 (50/81)
T567	2 or 3	32 (13/41)	0 ^a (0/28)	0 (0/13)
T655	2 or 3	59 (118/200)	86 (19/22)	66 (77/116)
T660	1 or 2	68 (71/104)	0 (0/11)	60 (42/70)
T711	1 or 2	68 (60/88)	11a (3/28)	67 (35/52)

^a Only seeds that failed to germinate were tested for GUS expression.

Fig. 3. Comparison of histochemical β-glucuronidase (GUS) activity in T90 transformants inoculated by either the wild-type *Mesorhizobium loti* strain NZP2037 or by the non-nodulating NodC mutant strain PN4047. **A**, **C**, **E**, and **F**, Development of GUS activity in roots and nodules of T90 plants 3, 7, 14, and 21 days, respectively, after inoculation with NZP2037. **B** and **D**, No evidence of GUS activity in roots of T90 plants 3 and 7 days, respectively, after inoculation with PN4047. Magnification: Bar = 0.5 cm.

Fig. 4. Inheritance of the transgene in progeny of the T90 line. Lanes 1–9, DNA from progeny of T90 cut with *Hind*III; lane 10, DNA from the primary transformant; and lane 11, wild type DNA from *Lotus japonicus*. Genomic DNA was hybridized with the 1.0-kb *Hind*III fragment immediately upstream of the T-DNA insertion in T90.

eny of T90. Figure 4 shows the result obtained with nine randomly chosen progeny, compared with the original primary transformant and a nontransformed plant. Two of the progeny were T-DNA homozygous (Fig. 4, lanes 2 and 7). Another two were heterozygous (Fig. 4, lanes 4 and 5), while five progeny (Fig. 4, lanes 1, 3, 6, 8, and 9) were identical to the wild type (Fig. 4, lane 11). There was complete agreement between presence of T-DNA and histochemical GUS activity, demonstrating a clear association between GUS expression phenotype and presence of T-DNA. The T-DNA homozygous individuals allowed us to determine that these plants showed no obvious nodulation or N₂ fixation phenotype, the latter of which was determined by the plants' ability to grow without access to mineral nitrogen.

The nucleotide sequence of the cloned 1-kb *L. japonicus* DNA fragment upstream from the T-DNA insertion did not suggest the presence of open reading frames encoding polypeptides of any significance, and searches for likely coding sequences, with the GENSCAN and TESTCODE programs, were negative. Furthermore, when this fragment was

used as a probe in a Northern (RNA) hybridization analysis of RNA from roots, nodules, and shoots of *L. japonicus*, no signal was detected (data not shown). However, the T-DNA insertion could be located in the promoter region of a gene. With this in mind, cloning of *L. japonicus* DNA downstream from the T-DNA insertion was attempted with uneven PCR (Chen and Wu 1997). With this method, a 1.7-kb fragment immediately downstream of the 1-kb *L. japonicus* fragment described above was amplified and cloned. Analysis of its sequence identified an open reading frame toward its 3' end with a predicted amino acid sequence that had strong homology to calcium-binding proteins, including calmodulins from a diverse range of organisms. However, only the sequence encoding the first 50 amino acids of the predicted peptide was present in the 1.7-kb fragment. This fragment was used as a probe in Northern blot hybridization analysis of roots and shoots of *L. japonicus* harvested 7 days after inoculation with wild-type *M. loti* NZP2235. A signal of approximately 800 bp was present in RNA from the roots whereas no signal was detected in RNA from the shoot (data not shown).

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AGGATAGTGAGTTGTGCTCAGCTCATATATTTACACCTCACTTTTTCTTTCTCATTTTCACCTTATTTTTCTTCTATCTTTCTCTGCAT      90
TTTCTGTACATCACATATCATATCACTTATTTCTCTAACTTCTCTTACATATACACAAGGTGAAAGTAAAAATTGATAGTAAACACAA      180
CATTACTCTTATATATACACACACTTTGTGGGCGATTATGTATGTGTGGTTCTAAATTAACTCGGTCCCTCTACCTTAGGCGGATTAG      270
AACAAGAGTTACACCTTTATCCTTATGAAAGAGAAGTGAGCATCTTGAATTGTTTCATGTACCCAAACATAATGAAACTATTAGGTTT      360
AGGTCTCCCTTCTTGGGAGGTCTAGACCTAGTCTTGTGTTTCAACAGTGAACACAACTTACCAACTTACCTTTAATGGGGTTAAATTAC      450
CCCATGGAGTAAATAGACAACCAAAAATTAAATACCTTTGAAAAATAAGCAACTAATTAACCTTGGGTTGTCTCAATTGGCCTCAGGTT      540
TTAAGTTAACTCTTATAATCTCATAGAGCGAGGTTCAAATCTACATCCAATAAAATCTCAGTTAATTGGTGTGTAATTCTCTGAG      630
ATCCTTTGGCGCTAGCTCTTACCTGAGAGTCAGCGTCTATTGATCTCCTCAGCATGTGACTAGCGTCATATCCAATAGTATGAAGGGAAT      720
AAGTGTGAACCACTGTATAAATCCCATCAGCAACATAAAATAAAATTTGATGATTTTTCTCATAATAGTGGCATATGAAATGTTGGA      810
AATGCAATAATTTTCTCTTGAGAAAAAAAATTGAAGATCCCAACAAGATGCCAGGTGTTGGCAGCATGAGTGAGCACGCGCAATAGCC      900
GTTGAGGAAATTCAAGTGACTAAAGTCAGTGCCTAATAAATAAATGCCGGCTTTACCCTCGTTATTTGGCGGGTCCACTCTCCAACG      990
TCTTCTATAATTTACTTTAGATCCGTCATCAACGGCCACTACCAATCTGTAAGGTTTATTAAGTCAATTTTCTACATTAAAAACCT      1080
TTCATAAAATAAACTTCATATAAAGACTAATTACATAAATAGGAGGAAAAATATATATACTCCATGACTCCATGTAATAAAAAATCCCGT      1170
GAAGGAACTGATAAAATATCTGATCTTTTGATCACTGATGAAAAATATATATCGTACCCTCTACGAAATTTTACAGCAACCAAGTC      1260
AGTCCAATTTTGTGTTGCTCATGATTGGAAAAATATCCTTTAAAGATATGTTTGTCCCGTCTTACAAGATTACTTGAGCAACCTGTATCC      1350
TCACACACAAGCTTCAAAAAAATAAACTTACGTTCACTTTACACCACAGAATTAGTCTTACAATTACATACTTAAAAATAATCAATT      1440
AAAAATTAATATTTCCACAAGAGTAGACCCCTTCAGAACAGGGGCATTAGACCGGTTGGGCAGAGTATTCTCTCTTTTTTTTATAGCTAA      1530
TGTCCATTAATTGAGTTATCTTAACACTTTTACACTCCACATCTTTGAGGACGGATAGAATTAAAGAAAAAGAAATGAATGTTATATATG      1620
TGATGTGATAGGAAAGTGAAGGAGAAACAGAAAGAAAAATTAAAGTAAAAATAAATTGTAAGAGAAAGAGGAATGTGAACCATAGTATAAA      1710
TCAATTATAATCATGAATTAAGTCAGTGGTAAGTTAACCAATAACTCACACAGCAAAATGTAGCCATGTAGGGCATCACAATTAAAGTGA      1800
CACGGGCCAACTTAAATACCTGCCAAGCAATTATTTAACGTGAACGCGTTTGTGAACAACCAAGTTCTCCTACTAGTTAATAAACAAAT      1890
GATTGTTTACCACCATAATTAGTGTAAAGTGCCATTAAATTAAACCTCCCAACACGCGTTTAAAGTGCCACACAGACAGCTAGCTATT      1980
GTTGCCCTTTAAAGCTTACCACCCTTCTCTCTGCTCAATCTAACAACAACAAAGAACCTCTTCTTCTCTATCTTTATCCACAA      2070
TTTTCACTCCCAAAATTTAGACAACAGACACCACCAAACTCTCCCAATGCCAACTATTTTGCATAGGATTTTCTCTTTTACAACTCC      2160
TAAATTCATTCTCTCTCTCTTGGTCCCCAAGAGGTGATAGCCTTCTCCCCCAATCTTGGTTCCTCCCATCAAACCCCTCTCTTTTCC      2250
TCCTCTCTCATCGTCATCATCAAGGGGGAACTTGTAAACAAAAACAACAGACGACTGTGGACCCCTGCCGGCTCTCTCCCTTTAGA      2340
TACGTCCTTAATACCAAAATGGACCCACCGAGCTCAAGCGTGTTCCTCAATGTTTGTAGAAACGGGGATGGCCGGATCAGCAAAAA      2430
M D P T E L K R V F Q M F D R N G D G R I T K K
AGAGCTCAATGACTCCCTTGGAATCTTGAATTTTCATACCTGACAAGGAGCTGACCCAGATGATCGAGCGGATTGATGTGAACGGAGA      2520
E L N D S L E N L G I F I P D K E L T Q M I E R I D V N G D
TGGGTGTGTCTACATTGATGAATTCGGGAGCTTTACCAGTCCATCATGGACGAGCGGATGAGGAGAAGGACATGAAGGAGGCTTTCAA      2610
G C V Y I D E F G E L Y Q S I M D E R D E E K D M K E A F N
CGTCTTCGATCAGAACGGGATGGCTTCATCCGTTGGAGGAGTTGAGGACCGTTCTGGCTTCACTCGGGATCAACAGGGGAGAACCGT      2700
V F D Q N C G D G F I T V E E L R T V L A S L G I K Q G R T V
GGAAGATTGCAAGAAGATGATCATGAACGTGGATGTTGATGGAGATGGTATGGTGGATTATCACGAGTTCAAGCAAATCATGAAAGGTGG      2790
E D C K K M I M N V D V D G D G M V D Y H E F K Q I M K G G
TGGCTTTAGCGCTCTCACTTAAACACAATTGATCATTTTCATTGATTCACTTCCCACGTTGTACATAAGTTTGGGGCATCTAGGTT      2880
G F S A L T *
GTTGCTGTACCACCAAGACCAACACAATCCCTGAACTAAAACCTCATCTCAATCTAACTATGCAATGTGTACTGTTACACAACCTTTTG      2970
TATTATGTGGCTTACCGAATTTGTACTATTGGGCCAGGCGACCATGACCCGAACGGGTCAAACACCATAATATATAAGGAGGGACAC      3060
CGCCCATGCGGTGGGGGAAT      3080

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Fig. 5. Nucleotide sequence of a 2.3-kb genomic sequence from *Lotus japonicus* upstream of a cDNA encoding a predicted 150 amino acid polypeptide homologous to calcium-binding proteins. The two bases in bold indicate position of the T-DNA insertion in the T90 line. The 2.3-kb genomic sequence has been deposited in the EMBL data base as accession no. AJ251807 and the cDNA encoding the polypeptide as accession no. AJ251808.

We used reverse transcription (RT)-PCR to isolate the remaining 3' end of the gene with RNA from the roots. The gene-specific forward primer was designed from the 5' end of the predicted coding sequence. An anchored poly-dT sequence was used as the 3' primer. An amplified product of approximately 750 bp was isolated, cloned, and sequenced. A polypeptide of 150 amino acids with a molecular mass of 17 kDa was deduced from the nucleotide sequence. The entire 2.3 kb upstream (possibly regulatory) sequence and the predicted coding sequence are shown in Figure 5, and the position of the T-DNA insertion is highlighted. Since the transcriptional start site has not yet been identified, we have made no attempt to identify potential TATA boxes and other regulatory elements. A TFASTA search of the data bases confirmed the strong homology of the predicted amino acid sequence to those of calmodulins and other calcium-binding proteins from many different types of organisms. The strongest similarity (73.9% identity) was found to a calcium-binding protein isolog predicted from a genomic sequence of *A. thaliana* (Fig. 6). The presence of common structural motifs, the "EF hands," is characteristic of calcium-binding proteins. These domains each bind a single Ca^{2+} ion (Snedden and Fromm 1998; Zielinski 1998). Four such motifs were identified in the cDNA from *L. japonicus* by searching motif data bases. The domains are highlighted in Figure 6. We therefore propose *LjCbp1* (calcium-binding protein) as the name for this gene. The consensus of these four domains (DXD/NGD/NGXV/IXXXE) is in good agreement with those of calcium-binding proteins from calmodulins and other calcium-binding proteins (DXD/NXD/NGXV/ISXXD/E) (Jang et al. 1998; Snedden and

Fromm 1998; Zielinski 1998). Most calmodulins and calmodulin-like proteins have four Ca^{2+} -binding domains, although numbers can vary between 3 and 6 (Snedden and Fromm 1998; Zielinski 1998).

Expression and genomic organization of *LjCbp* in *L. japonicus*.

Expression of the *LjCbp1* gene in roots and shoots of *L. japonicus* was investigated by Northern blot hybridization. The results are summarized in Figure 7. No expression was detected in shoots, whether the roots were nodulated or not. The strongest signal was found in roots of plants inoculated by wild-type *M. loti*. Substantially lower expression was found in roots inoculated by the NodC mutant PN4047. The expression was also lower in roots of T90 plants that were homozygous for the T-DNA insertion, compared with Gifu (wild-type) *L. japonicus*. This was the case whether inoculated by wild-type or NodC mutant *M. loti* strains. RT-PCR with primers specific to *LjCbp1* confirmed that no expression was detected in RNA from the shoots, but expression was found in roots of both Gifu and heterozygous and homozygous T90 plants (data not shown). Overall, the expression of *LjCbp1* had a pattern similar but not identical to that of the GUS expression observed in T90 plants. In both cases, no expression was seen in shoot material, levels of expression decreased in nodulated roots with the age of the plants, and expression was gradually confined to nodules alone. An apparent discrepancy was seen in roots inoculated by the NodC mutant of *M. loti*. No GUS activity was detected, whereas expression of *LjCbp1* was evident in North-

	10	20	30	40	50	60
	.	.	.	.	.	.
		.E K F .FD. GDG IT EL. . LG . .L MI				
Lj Cbp	-----MDPTELKRVFQMF	<u>DRNGDGRITKKEL</u>	NDNSLENLGIFIPDKELTQMI			
At Cbp	MLPSPSSSSAPT	KRIDPSELKRVFQMF	DKNGDGRITKEELNDNSLENLGIYIPDKDLTQMI			
Pv Cam	-----MADQLTDEQISEFKEAFSLFDKDGDCITTTKELGTVMRSLGQNPTAEALQDMI					
Rn Cam	-----MADQLTEEQIAEFKEAFSLFDKDGDTITTTKELGTVMRSLGQNPTAEALQDMI					
	70	80	90	100	110	120
	.D G G. . EF E . . .AF VFD. G.I. EL.					
Lj Cbp	ERIDVNGDGC	<u>VYIDEF</u>	GELYQSIMDERD-----EEKDMKEAFNVFD	<u>QNGDGFITVEEL</u>	RT	
At Cbp	HKIDANGDGCVD	IDEFESLYSSIVDEHHNDGETEEEDMKDAFNVFDQDGDGFITVEELKS				
Pv Cam	NEVDADGNGTIDFPEFLNLMARKMKD	TD-----SEEE-LKEAFRVFDKQNGFISAAELRH				
Rn Cam	NEVDADGNGTIDFPEFLTMMARKMKD	TD-----SEEE-IREAFRVFDKDGNGYISAAELRH				
	130	140	150	160	170	
	V. . LG K T . MI D DGDG . Y EF . .M					
Lj Cbp	VLASLGIKQGR	TVEDCKKMIMNV	<u>DVDGDGMVDYHEF</u>	KQIMKGGGFSALT-		
At Cbp	VMASLGLKQGK	TLDGCKKMIMQVDADGDGRVNYKEFLQMMKGGGFSSN				
Pv Cam	VMTNLGEK--	LTYYEVD	EMIREADV	DGDGQINYYEEFVKVMMAK-----		
Rn Cam	VMTNLGEK--	LTDEEVD	EMIREAD	IDGDGQVNYEEFVQMMTAK-----		

Fig. 6. Comparison of deduced amino acid sequence of *LjCbp1* to full-length sequences of other calcium-binding proteins. *AtCbp* is a "calcium-binding protein isolog" from *Arabidopsis thaliana* (EMBL accession no. O22845), *PvCaM* is a calmodulin from *Phaseolus vulgaris* (EMBL accession no. AF030033), and *RnCaM* is a calmodulin from *Rattus norvegicus* (EMBL accession no. M16659). The four calcium-binding domains are highlighted. The sequence of *LjCbp1* has been deposited in the EMBL data base as accession no. AJ251808.

ern blot hybridization analyses, albeit at reduced levels compared with plants inoculated with wild-type *M. loti*.

Southern blot analysis of *L. japonicus* genomic DNA with a probe from the noncoding region upstream of the T-DNA insertion suggested the presence of a unique DNA sequence (Fig. 4). By contrast, with the *LjCbp1* cDNA clone as a probe, multiple hybridizing fragments were found even after stringent washing of the filter (Fig. 8), suggesting that a family of closely related genes encoding calcium-binding proteins is present in *L. japonicus*.

DISCUSSION

The results presented here show that promoter tagging in the model legume *L. japonicus* can be used to identify tagged lines specifically expressing the *uidA* reporter gene in roots and root nodules. Germinating embryos of four of the seven GUS-positive tagged lines also expressed GUS. These four lines all had more than one T-DNA insertion, so this observation might be explained by different copies of T-DNA being responsible for GUS expression in the different tissues. Analysis of segregation of T-DNA copies in the progeny will determine whether this is the case.

The distinctive GUS expression patterns observed in roots and nodules of the seven tagged lines described here were inherited in the T₂ progeny. This demonstrates the stability of T-

DNA integration into the plant genome. It was, however, surprising that in most of the GUS-positive tagged lines expression of the reporter gene was root and/or nodule specific, and that no tagged lines with GUS expression in other plant tissues were found in the initial screen. This would suggest either that the number of plant genes that are switched on in nodules has been underestimated considerably (Stougaard Jensen et al. 1986) or, more likely, that T-DNA insertions into transcribed genes are not entirely random. The results of Martirani et al. (1999) suggest that the *L. japonicus* transformation system with *A. rhizogenes* can be used to increase further the number of integration events into root-expressed genes.

In the T90 line, the T-DNA insertion is positioned about 1.3 kb upstream of a gene encoding a polypeptide homologous to calcium-binding proteins (Fig. 5). The fact that we observed no obvious nodulation or N₂ fixation phenotype could be explained in several ways. The first alternative is that the T-DNA insertion did not interrupt the *LjCbp1* coding sequence, thereby permitting sufficient residual expression and synthesis of the gene product to produce a normal phenotype. RNA from the roots of this plant line did indeed hybridize with a *LjCbp1* probe (Fig. 7). The second alternative is that expression of *LjCbp1* is completely knocked out by the T-DNA, and the hybridizing signal is due to expression of a homologous gene, providing functional complementation. The Southern data (Fig. 8) showed that several genes, strongly homologous to *LjCbp1*, are present in the *L. japonicus* genome. However, RT-PCR amplification of a product from homozygous T90 root RNA with *LjCbp1*-specific primers (data not shown) suggests that the former explanation is most likely. Third, *LjCbp1* may not be essential for establishing an effective symbiosis. The early nodulin ENOD12 is one example of a gene product that is apparently not essential for formation of effective nodules in *Medicago sativa* (Csanadi et al. 1994), despite being elicited by a Nod factor (Journet et al. 1994).

In contrast to the coding sequence of *LjCbp1*, the upstream sequence appears to be present as a single copy in *L. japonicus* (Figs. 2 and 4). It is becoming clear that many plant spe-

Fig. 7. Expression of *LjCbp1* mRNA in roots (R), and shoots (S), of *Lotus japonicus* wild-type plants (Gifu), or T90 transformants homozygous for the T-DNA tag, inoculated with either wild-type (NZP2037) or non-nodulating mutant (PN4047) of *Mesorhizobium loti*. Seven micrograms of total RNA was loaded in each lane. Hybridization with *LjUbiquitin* probe was included for normalization.

Fig. 8. Genome organization of *LjCbp1*. Southern hybridization of DNA from *Lotus japonicus* with cDNA probe of *LjCbp1*. E1: *EcoRI*; H: *HindIII*; EV: *EcoRV*.

cies have calmodulin multigene families consisting of several identical copies of calmodulin and other calmodulin-related genes (Snedden and Fromm 1998; Zielinski 1998). The presence of multiple copies of *LjCbp1*, or very closely related genes, and a unique upstream promoter sequence is consistent with the idea that each member of the gene family has a different temporal and spatial expression pattern, but that all perform the same function (Snedden and Fromm 1998). However, further work is needed to test this possibility. Analysis of the regulatory properties of the 2.3-kb upstream sequence will clarify its role in controlling *LjCbp1* gene expression.

The fact that *LjCbp1* expression was lower in the T90 homozygous line than in the wild-type plants indicates that the T-DNA insertion did affect expression of this gene. The absence of visible GUS activity in T90 plants inoculated with the NodC mutant strain of *M. loti* is in apparent contrast to the reduced levels of *LjCbp1* gene expression in both T90 and untransformed *L. japonicus* plants inoculated by the NodC mutant (Figs. 1, 2, and 7). However, we have not tested the possibility that the *uidA* gene is expressed at a very low level in these plants. The identification of a gene encoding a calcium-binding protein, the expression of which is enhanced by nodulating rhizobia but not (or weakly so) by rhizobia that do not produce Nod factor, may prompt speculation that this gene is involved in the early signal transduction processes involved in nodulation. Nod factors elicit transient and localized increases in the concentration of cytosolic Ca^{2+} in the root hairs of legumes (Ehrhardt et al. 1996; Gehring et al. 1997; Felle et al. 1998, 1999; de Ruijter et al. 1998; Cárdenas et al. 1999). These increases in cytosolic Ca^{2+} appear to be crucial for transduction of the Nod factor signal (Felle et al. 1999). A hypothetical model has been proposed for the Nod factor signal transduction pathway involving G protein-mediated influx of Ca^{2+} either from outside the plasma membrane, or via inositol-1,4,5-triphosphate, from internal stores (Pingret et al. 1998). A number of cDNAs encoding GTP-binding proteins have been identified in *L. japonicus*, some of which had elevated expression levels in root nodules (Borg et al. 1997). Whatever the mechanism of signal transduction, it appears that Nod factors elicit changes in the cytosolic Ca^{2+} concentration in root hairs, and that Ca^{2+} is an important second messenger in signaling pathways, often mediated by calcium-binding proteins. It seems likely that such proteins are involved in the Nod factor-induced signaling, since calmodulins and calmodulin-related proteins play important roles in other signal transduction processes in plants. Both biotic and abiotic external signals elicit transient increases in the cytosolic Ca^{2+} concentration. By responding to the increased Ca^{2+} concentration in the cytosol, calmodulin-related proteins are structurally modulated, enabling them to interact with a variety of targets (Liao et al. 1996; Ling et al. 1994). They, in turn, control many different cellular functions. Some of these include protein modifications and changes in the cytoskeleton (Snedden and Fromm 1998; Zielinski 1998; Trewavas and Malhó 1997, 1998). Nod factors also elicit rearrangement of the actin filaments in the cytoskeleton of root hair cells (de Ruijter et al. 1999). The likely calcium-binding properties of the *LjCbp1* gene product identified in this work make it a potential candidate for involvement in the signal transduction that takes place in response to Nod factors and triggers the processes that eventually leads to formation of root nodules. However, before we

can draw any conclusions about the role of the putative calcium-binding protein *LjCbp1* in the symbiotic interaction with *M. loti*, much more work is needed. First, we need to establish whether the gene product actually binds Ca^{2+} . Second, it will be necessary to obtain a more detailed picture of the temporal expression pattern of *LjCbp1* prior to 3 days post inoculation, and in particular to ascertain whether a Nod factor is sufficient to induce expression of this gene. Some of the effects of Nod factors are seen within minutes (Cárdenas et al. 1999; Felle et al. 1999; de Ruijter et al. 1999), which suggests that de novo gene expression is not involved in this early response to Nod factors.

MATERIALS AND METHODS

Transformation.

L. japonicus (Regel) Larsen cv. Gifu B-129-S9 generation was provided by J. Stougaard (University of Aarhus, Denmark). Seedling hypocotyls were cut longitudinally and inoculated with *A. tumefaciens* (strain LBA4404) containing the vector p Δ gusBin19 (Topping et al. 1991), according to Handberg and Stougaard (1992). This binary vector contains a promoterless *uidA* gene at the T-DNA left border and an antibiotic, selectable, marker gene (*nptII*, which confers resistance to geneticin and kanamycin) at the right border of the T-DNA. Transformed callus was selected on 25 mg liter⁻¹ geneticin (Gibco BRL, Paisley, UK) by transferring to fresh selection medium every week until green callus was produced. Non-transformed control plants were produced by regenerating seedling hypocotyls as above without the inoculation or selection stages. Once rooted, the plants were transferred to a mixture of peat and soil (1:1) and grown in a controlled environment room at 20°C, light intensity 400 $\mu\text{E s}^{-1} \text{m}^{-2}$ with a 16-h photoperiod. Plants derived from the same callus, which may therefore have resulted from the same transformation event, were given the same line number, followed by letters A or B. For example, T90A and T90B are different plants derived from the same callus. Plants derived from different calli are referred to as independent transformants as they resulted from different transformation events.

Analysis of roots of primary transformants and seedlings.

Five replicated shoot cuttings of each primary transformant were placed in an aerated, hydroponic system containing 2 liters of nitrogen-free nutrient solution (Ryle et al. 1978). Once roots appeared, the cuttings were inoculated with *M. loti* NZP2235 (Jarvis et al. 1982). Bacteria were grown on YM agar slopes (Vincent 1970) at 28°C for 24 h, dispersed in 5 ml of sterile tap water, and added to the nutrient solution of the hydroponic system. Roots without nodules were grown in nutrient solution without *M. loti* and supplemented with 10 mM potassium nitrate. Roots were tested for the presence of GUS activity by histochemical staining (as described below), at four stages of root and root nodule development: (i) before root nodule development; (ii) roots with white immature nodules (before the production of leghemoglobin); (iii) roots with pink mature nodules (containing leghemoglobin required for nitrogen fixation); and (iv) in roots with senescent nodules. Senescence was induced in nodules by growing nodulated cuttings in the dark for 10 days. Seeds were germinated in vermiculite with a nitrogen-free nutrient solution, and inocu-

lated with *M. loti* NZP2037 (Chua et al. 1985) or PN4047 (Chua et al. 1985; Scott et al. 1996) after 7 days. Seedlings were harvested for histochemical localization of GUS activity.

Histochemical localization of GUS activity in plant tissues.

All transformed tissues were tested for GUS activity by incubation in 1 mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronide (Melford Laboratories, Suffolk, UK) at 37°C for 5 to 7 h. The tissues were cleared in glacial acetic acid and absolute ethanol (3:1, vol/vol) for 24 h and fixed in lactic acid, water, and glycerol (1:1:1, vol/vol/vol). Samples were examined under stereo light microscopy for blue staining. Photographs were taken on Kodak Ectachrome Elite film with an Olympus microscope and camera.

Detection of transgenes in plants by PCR.

The presence of the *nptII* and *uidA* genes in primary transformants and their progeny was detected by PCR. DNA extracts were prepared from two young leaves according to Edwards et al. (1991). PCRs were as described by Webb et al. (1999). The *nptII* gene was amplified with the primer pair designed by Hamill et al. (1991): 5'-GAGGCTATTCGGCTATGACTG-3', and 5'-ATCGGGAGCGGCGATACCGTA-3' to produce a product of 0.7 kb. The *uidA* gene (Jefferson et al. 1986) was amplified with a primer pair designed with the Prime program (Wisconsin Package version 9.1; Genetics Computer Group [GCG], Madison, WI): 5'-TAGCGGGACTTTGCAAGTG-3' and 5'-GTTTTGCAGCAGAAAAGCC-3', producing an amplified product of 1,066 bp.

Southern and Northern hybridizations.

For Southern blot hybridizations, DNA from *L. japonicus* was extracted by the method of Handberg and Stougaard (1992). Ten micrograms of DNA cut with the appropriate restriction endonuclease was separated by agarose gel electrophoresis, and transferred to Nylon membranes (Amersham Life Sciences, Little Chalfont, UK) by capillary transfer (Sambrook et al. 1989). Hybridizations with [³²P]dCTP-labeled DNA probes were carried out at 65°C. Membranes were washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate (SDS) for 20 min at 30°C, once with 0.2× SSC, 0.1% SDS for 20 min at 65°C, and once with 0.1× SSC, 0.1% SDS for 20 min at 65°C.

From plant material up to 14 days old (7 days after inoculation with *M. loti*) RNA was extracted with TRIzol reagent (Gibco BRL, Paisley, UK) as described by the manufacturer. RNA from older plant material was extracted according to Verwoerd et al. (1989). For Northern analyses, RNA was separated by agarose/formaldehyde gel electrophoresis (Sambrook et al. 1989), and hybridization was carried out as described by Skøt et al. (1997). Filters were washed twice with 2× SSC, 0.1% SDS at 30°C for 20 min; once with 0.2× SSC, 0.1% SDS at 42°C, and once at 65°C for 20 min each.

Inverse PCR, uneven PCR, and RT-PCR.

DNA for inverse PCR and uneven PCR was extracted according to Handberg and Stougaard (1992). Inverse PCR was used to amplify and clone plant DNA flanking the T-DNA insertion in the T90 line. This was done essentially as described by Lindsey et al. (1993). Genomic DNA was completely cut

with *Hind*III, and ligated under conditions favoring circularization. Amplification was carried out with two primers from the *uidA* gene: 5'-CAGGACGTAACATAAGGG-3' and 5'-GACTGGCATGAACTTCG-3'.

Uneven PCR was used to amplify DNA from wild-type *L. japonicus* downstream from the 1-kb *Hind*III fragment cloned by inverse PCR as described above. Uneven PCR was developed by Chen and Wu (1997) as a tool to clone unknown DNA adjacent to known sequences. It uses a gene-specific oligonucleotide as one primer and an arbitrary short oligonucleotide as the other primer in PCR. In the PCR, the annealing temperature alternates between high and low in consecutive cycles to optimize the yield of specific products rather than nonspecific ones. The sequence of the gene-specific primer was as follows: 5'-TGAAGATCCCAACAAGATG-3'. This primer was used in 12 amplification reactions each with a different random 10mer. The 12 random primers were selected from a random amplified polymorphic DNA (RAPD) kit (Advanced Biotechnologies, Epsom, UK). The only constraint was that each primer had a G+C content of 60%. The cycling conditions for the first round of amplification were 94°C for 1 min, then for cycle 1 94°C for 30 s, 52°C for 1 min, 72°C for 1 min, and for cycle 2, 94°C for 15 s, 42°C for 1 min, and 72°C for 1 min. Cycle 1 and 2 were conducted three times. For cycles 7 and 8 conditions were 94°C 15 s, 57°C 30 s, 72°C 30 s, and 94°C 15 s, 45°C 30 s, 72°C 30 s. Those two cycles were conducted 20 times followed by 72°C for 5 min. The reaction volume was 50 μ l and contained 30 ng of target DNA, 200 μ M of dNTPs, 0.25 μ M of the gene-specific primer, 0.05 μ M of random primer, 1.5 mM MgCl₂, PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl) and 2.5 U of Amplitaq DNA polymerase (PE Biosystems, Warrington, UK). A 0.5- μ l aliquot of the product was used in a second round of uneven PCR. The components were the same except that a nested, gene-specific primer was used: 5'-TGTTGGCAGCATGAGTGAG-3'. The cycling conditions were 94°C for 1 min, then for cycle 1, 94°C 15 s, 57°C 30 s, 72°C 30 s, and for cycle 2, 94°C 15 s, 45°C 30 s, 72°C 30 s. Those two cycles were conducted 20 times, followed by 72°C for 5 min. A Geneamp PCR System 2400 (PE Biosystems) thermal cycler was used. Amplification products were visualized by agarose gel electrophoresis.

The Titan One Tube RT-PCR system (Roche Diagnostics, Lewes, UK) was used according to the manufacturer's instructions to obtain the cDNA encoding *LjCbp1*. The gene-specific forward primer included the putative start codon and was 5'-CGTCCCTAATACCAAAAATG-3'. The sequence of the reverse primer included a poly-dT tail, and was 5'-GACTCGAGTCGACATCGAT₁₇-3'. The annealing temperature for the reaction was 55°C.

Cloning, nucleotide sequencing, and analysis.

The cDNA product derived from RT-PCR and those resulting from inverse PCR and uneven PCR were cloned in the pGEMT-Easy vector (Promega Life Sciences, Southampton, UK) according to the manufacturer's instructions. Nucleotide sequencing was carried out with the pGEMT-Easy-derived plasmids, with the "Thermo-sequenase dye terminator cycle sequencing kit" (Amersham Life Sciences). An ABI-373 sequencer (PE Biosystems) was used for sequence detection. The sequences were assembled with the Wisconsin Package version 9.1 (GCG, Madison, WI). Searches for open reading

frames, likely coding sequences, and gene predictions were carried out with GRAIL (Xu and Uberbacher 1997), GENESCAN (Burge and Karlin 1997), TESTCODE (Wisconsin Package version 9.1; GCG), and NetStart 1.0 (Pedersen and Nielsen 1997). Secondary structures and motifs were identified with PredictProtein (Corpet et al. 1998), and GenomeNet (Institute for Chemical Research, Kyoto University, Japan).

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