*TECHNICAL ADVANCE*

# Polyubiquitin Promoter-Based Binary Vectors for Overexpression and Gene Silencing in *Lotus japonicus*

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**In this study, we compared the transcriptional activities between *Cauliflower mosaic virus* (CaMV)35S promoter and polyubiquitin (*Ljubq1*) promoter from *Lotus japonicus* using β-glucuronidase *(gus)* reporter gene in transgenic plants of *L. japonicus*. The promoter analysis demonstrated that the *Ljubq1* promoter possessed higher activity than the CaMV35S promoter in leaves, stems, roots, nodules, and pollen. Finally, we created GATEWAY conversion technology-compatible binary vectors for over-expression and RNA interference under the *Ljubq1* promoter. These materials could provide alternative choice for studies in *L. japonicus*.**

Strong promoters have been used for various applications in molecular biology, such as overexpression and RNA interference (RNAi). In addition to the high transcriptional activity, ubiquitous expression in most cell types often is demanded for these applications. In plants, the 35S promoter from *Cauliflower mosaic virus* (CaMV35S promoter) fulfills these requirements in several species. However, in *Lotus japonicus*, although used for the molecular studies of root nodule symbiosis (Handberg and Stougaard 1992; Udvardi et al. 2005), the activity of the CaMV35S promoter in nodules generally is shown to be weak in other species (Auriac and Timmers 2007; Obertello et al. 2005; Quandt et al. 1993; Samac et al. 2004).

As an alternative to CaMV35S promoter, the high transcriptional activity of polyubiquitin promoters has been investigated in plants. Ubiquitin is involved in protein degradation in conjunction with the 26S proteasome, which recognizes ubiquitin-conjugated proteins (Smalle and Vierstra 2004). The polyubiquitin gene encodes a tandem repeat of ubiquitin and is expressed in various tissue types of plant (Binet et al. 1991; Burke et al. 1988; Kawalleck et al. 1993; Wang et al. 2000). The genomic structure of polyubiquitin is conserved among various plants, including *Helianthus annuus* (sunflower), *Zea mays* (maize), *Petroselinum crispum* (parsley), *Arabidopsis thaliana*, *Nicotiana tabacum* (tobacco), *Solanum tuberosum* (potato), *Pisum sativum* (pea), *Oryza sativa* (rice), and *Gladiolus* spp. (Binet et al. 1991; Christensen et al. 1992; Garbarino et al. 1995; Genschik et al. 1994; Joung and Kamo 2006; Kawalleck et al. 1993; Norris et al. 1993; Wang et al. 2000; Xia and Mahon 1998). It has been reported that the well-con-

served intron located in the 5′ untranslated region (UTR) contributes to the high transcriptional activity in *A. thaliana*, potato, tobacco, and gladiolus (Garbarino et al. 1995; Joung and Kamo 2006; Norris et al. 1993; Plesse et al. 2001). In this study, we attempted to isolate the *L. japonicus* polyubiquitin promoter, whose activity was expected to be high and ubiquitous in *L. japonicus*, and we constructed the polyubiquitin promoter-based binary vectors.

## RESULTS AND DISCUSSION

### Isolation and gene structure of *Ljubq1*.

To identify a polyubiquitin gene, which potentially possesses the strongest transcriptional activity, we used datasets from The Gene Index Project of *L. japonicus* at the Computational Biology and Functional Genomics Laboratory of Dana-Farber Cancer Institute and cDNA Array Expression Profiles of *L. japonicus* (Kouchi et al. 2004). In the comparison of *Arabidopsis* polyubiquitin (UBQ3; AAO00780) to The Gene Index Project of *L. japonicus*, we found 13 tentative consensus sequences (TCs) encoding putative polyubiquitin. Among them, TC14054 consists of 235 expression sequence tags (ESTs) obtained from various tissues, including roots, nodulating roots, immature nodules, mature nodules, flower buds, flowers, pods, and young plants, whereas the others consist of 3 to 47 ESTs. This indicates that TC14054 is a highly and ubiquitously expressed polyubiquitin gene. The cDNA Array Expression Profiles supported this prediction. A polyubiquitin-encoding EST (AV422262), which is expressed the highest in roots, nodulating roots, nodules, and shoots, also belongs to TC14054. Consequently, TC14054 was termed as *Ljubq1*.

To obtain the genomic structure of *Ljubq1*, the sequence of the *L. japonicus* genomic clone LjT06D07, which included the sequences of *Ljubq1*, was analyzed. The structure of *Ljubq1* is shown in Figure 1A. The *Ljubq1* has two exons, separated by a 5′ UTR intron of 540 bp. The length of noncoding exon1 is predicted to be 47 bp based on 5′ end sequences of an EST (CB828077). A putative TATA box (TATAA) was found at the position of –39 to –43 bp from the putative transcription initiation site. The exon2 has an open reading frame of ubiquitin pentamer. Each ubiquitin monomer consists of 76 amino acids and shows 100% amino acid identity to every other monomer except for the fifth ubiquitin monomer, which has an additional phenylalanine residue at the C-terminal end. An intron at the 5′ UTR is located directly before the translation initiation codon of *Ljubq1*. The nucleotide sequences CAAG/gta at the

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5′ splice site and cag/ATG at the 3′ splice site were predicted. This structure was similar to the others as descried previously (Binet et al. 1991; Christensen et al. 1992; Garbarino et al. 1995; Genschik et al. 1994; Joung and Kamo 2006; Kawalleck et al. 1993; Norris et al. 1993; Wang et al. 2000; Xia and Mahon 1998).

**The promoter activity in roots and nodules of hairy roots.**

The promoter activities of four DNA fragments of *Ljubq1* were compared in hairy roots. The hairy root transformation is a rapid procedure to generate transgenic roots in *L. japonicus* (Hansen et al. 1989). The 535- or 1,118-bp fragments of *Ljubq1* promoter with or without the 5′ UTR intron were fused to the β-glucuronidase (GUS) gene by replacement of the CaMV35S promoter of pBI121 (Jefferson et al. 1987). These four vectors were termed pUB500, pUB1100, pUB500i, and pUB1100i (Fig. 1B).

The hairy roots transformed with four vectors of *Ljubq1* showed intense GUS staining in roots and inside of nodules (Fig. 2A to D), whereas the hairy root transformed with pBI121 showed GUS staining in roots and less staining inside of nodules (Fig. 2E). The averages of GUS activity were at almost the same level among four vectors of *Ljubq1* and approximately threefold higher than CaMV35S promoter in roots and nodules (Fig. 2G). Notably, the maximum activities of pUB500i and pUB1100i were higher than pUB500 and pUB1100, respectively (Fig. 2G), indicating that the 5′ UTR intron potentially enhanced the transcriptional activities. Taken together, we concluded that the 535-bp fragment of *Ljubq1* with the 5′ UTR intron was suffi-cient for a high-level expression of *Ljubq1*, even if the variability of GUS activity (Fig. 2G) and staining patterns (data not shown) for all constructs are taken in account.

**The promoter activity in leaves, stems, roots, nodules, and pollen of stable transgenic plants.**

To investigate the activities of the *Ljubq1* promoter at ground tissues, stable transgenic plants were generated. As $T_0$ generation, 58 and 73 lines were transformed with pUB500i and pBI121, respectively, and resulting in strong GUS activity in roots or leaves in 18 and 20 lines, respectively. Seed of 13 lines each were obtained from lines of pUB500i and pBI121 transgenic plants in the $T_1$ generation. We used the well-growing $T_1$ lines for the following GUS assay.

The GUS activities of pUB500i-transformed plants were higher than pBI121-transformed plants in all surveyed tissues (Fig. 3). Intense GUS staining was detected inside nodules of pUB500i-transformed plants (Fig. 3G) similar to that observed in the hairy-root experiments. The mature pollen of pUB500i-transformed plants showed strong GUS staining (Fig. 3M), whereas those of pBI121-transformed plants showed no GUS staining (Fig. 3N). The weakness of CaMV35S promoter in pollen has been reported in stable transgenic plants of *N. tabacum* (tobacco), *Petunia hybrida* (petunia), and *Lycopersicon esculentum* (tomato) (Guerrero et al. 1990; Mascarenhas and Hamilton 1992; van der Leede-Plegt et al. 1992). This result indicates that CaMV35S promoter is unsuitable for ectopic gene expression studies in *Lotus japonicus* pollen. The averages of GUS activity in leaves, stems, roots, and nodules of pUB500i-transformed
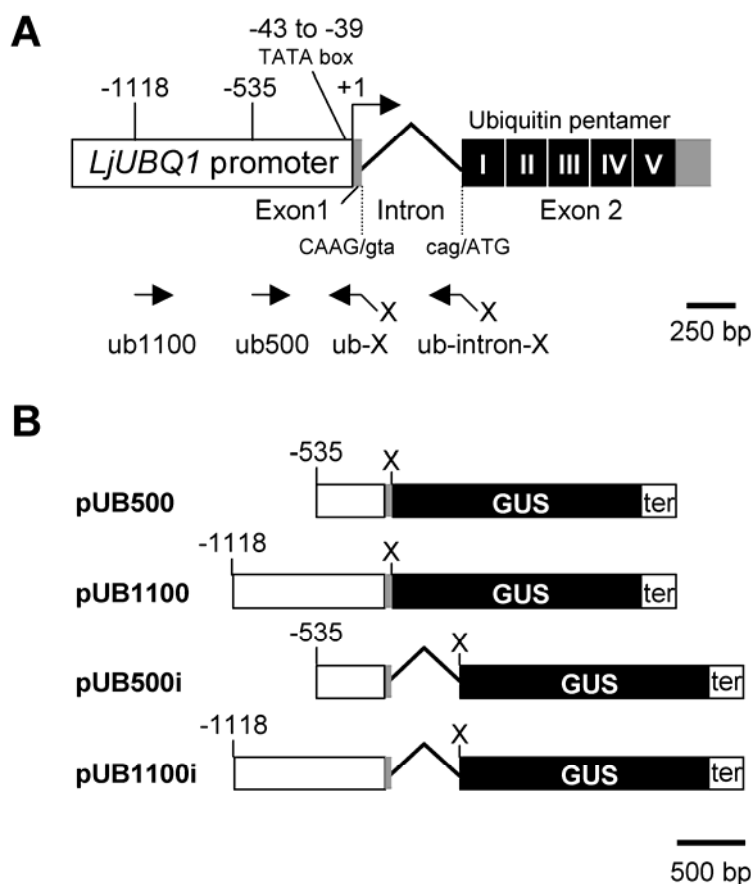


**Fig. 1.** *Ljubq1* structure and the binary vectors for β-glucuronidase (GUS) assays. **A,** Gene structure of *Ljubq1*. *Ljubq1* contains two exons separated by a 5′ untranslated region (UTR) intron of 540 bp. The noncoding exon1 is predicted to be 47 bp. Exon2 has an open reading frame of ubiquitin pentamer. The 5′ and 3′ UTRs are shown as gray box. A putative transcription initiation site is represented as +1 with an arrow. A putative TATA box (TATAA) is located at the position of –39 to –43 bp. The positions of primers to construct the binary vectors are presented below. **B,** Binary vectors for GUS assays. Additional bases were inserted in the end of *Ljubq1* fragments due to the *Xba*I (X) site of the primers; ter = *nos* terminator.

plants were approximately eight-, seven-, four-, and fivefold higher, respectively, than those of pBI121-transformed plants (Fig. 3P). These results indicate that the *Ljubq1* promoter is suitable not only for high-level expression in nodules but also for aboveground tissues of *L. japonicus*.

### *Ljubq1* promoter-based binary vectors for overexpression and gene silencing.

pCAMBIA1300 (Cambia, Canberra, Australia) was used as a backbone in the general structure of *Ljubq1* promoter-based binary vectors. pCAMBIA1300 is a derivative of *pPZP*. The features of *pPZP* are high copy replication in *Escherichia coli* and high stability in *Agrobacterium* spp. without antibiotics (Hajdukiewicz et al. 1994). For bacterial selection (in *E. coli* or *Agrobacterium* spp.), kanamycin is available. The plant-selectable marker gene (*hptII* for hygromycin resistant) is located next to the left border. Because the T-DNA transfer is initiated from the right border, the hygromycin resistance means that an entire T-DNA is transferred into plants. The hygromycin resistance gene is more suitable for transformation
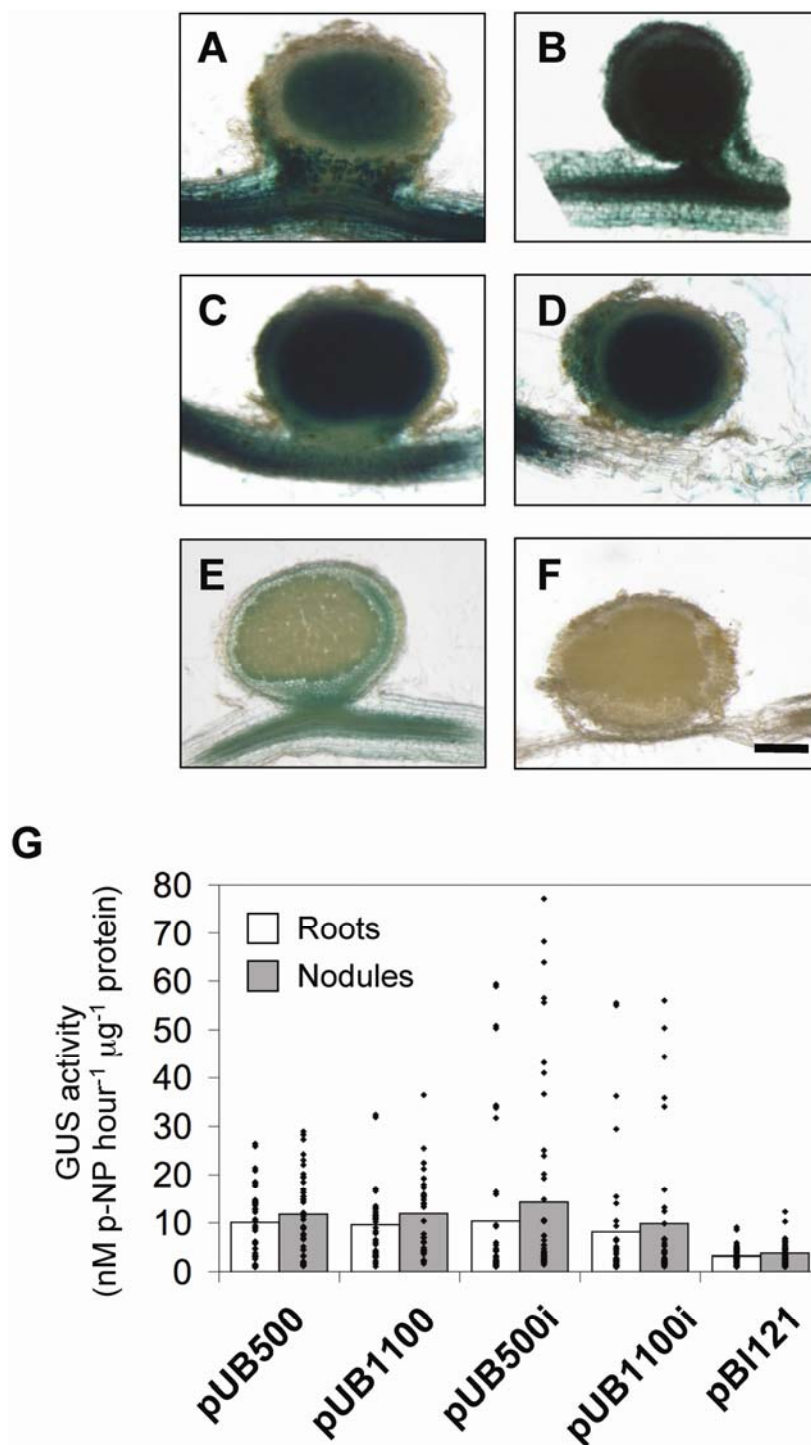


**Fig. 2.** Promoter activity in hairy roots. **A** through **F,** Pictures of β-glucuronidase (GUS)-stained nodule sections. Nodules were obtained from the plants transformed with **A,** pUB500; **B,** pUB1100; **C,** pUB500i; **D,** pUB1100i; and **E,** pBI121; and **F,** untransformed MG-20. **G,** GUS activity in roots and nodules. The white and gray bars represent the average GUS activity in roots and nodules, respectively ($n = 60$). Black dots represent individual values of GUS activity from independent roots. There are significant differences between pUB500i and pBI121 both for roots and nodules ($P < 0.01$). Bar = 250 μm.
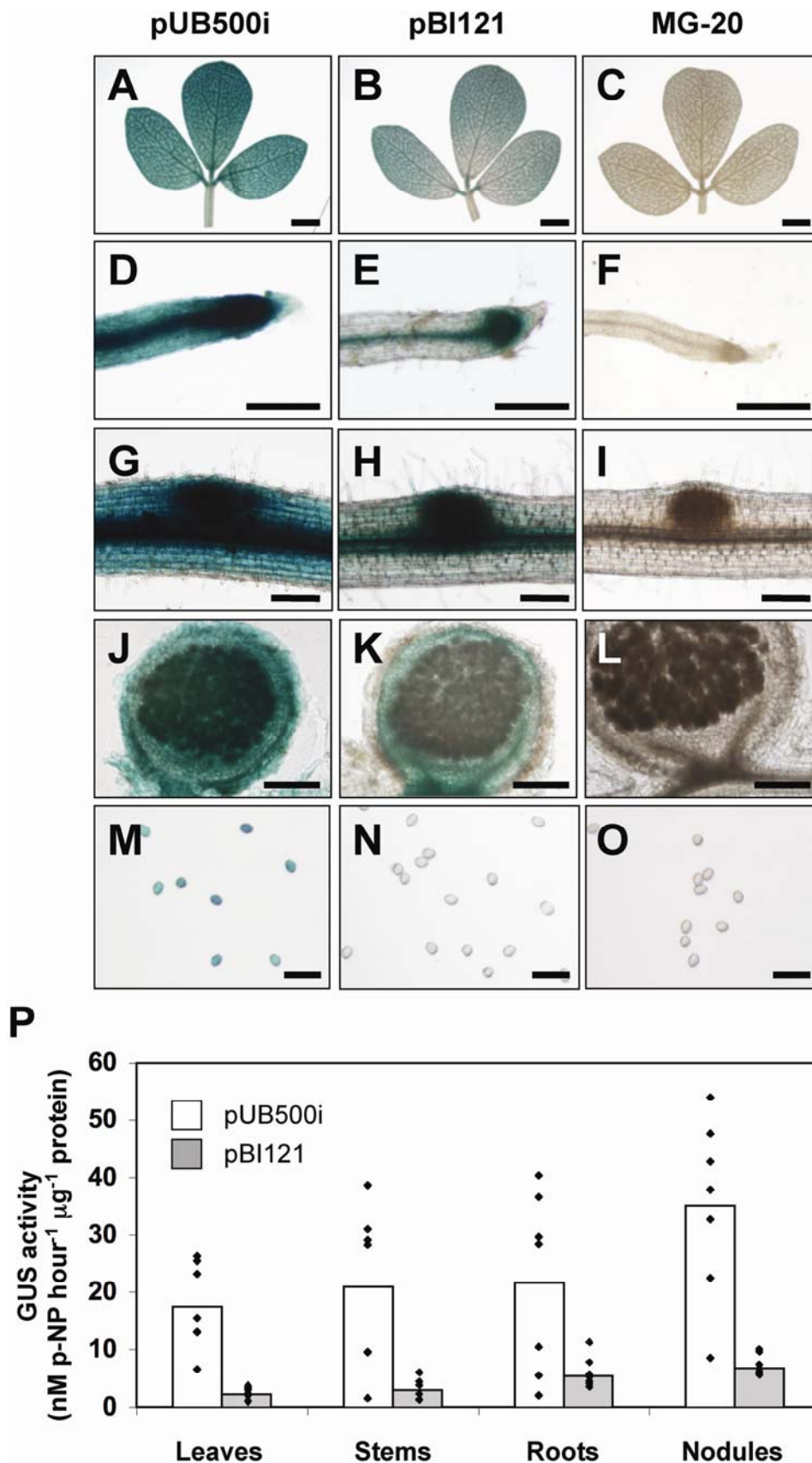
**Fig. 3.** Promoter activity in the stable transgenic plants. Pictures of β-glucuronidase (GUS)-stained **A** through **C,** leaves; **D** through **F,** root tips; **G-I,** nodule primordia; **J** through **L,** nodule sections; and **M** through **O,** mature pollen obtained from the stable transgenic plants (T$_1$) with **A, D, G, J,** and **M,** pUB500i; **B, E, H, K,** and **N,** pBI121; and **C, F, I, L,** and **O,** untransformed MG-20. **P,** GUS activity in leaves, stems, roots, and nodules. The white and gray bars represent the average GUS activity of each tissue transformed with pUB500i and pBI121, respectively ($n$ = 7). Black dots represent individual value of GUS activity from independent transgenic lines. There are significant differences between pUB500i and pBI121 for all combinations ($P < 0.01$; except for roots, $P < 0.02$). Bars = **A** through **F,** 1 mm; **G** through **L,** 250 μm; and **M** through **O,** 50 μm.

of *L. japonicus* than the kanamycin resistance gene because the hygromycin selection results in less escape than the kanamycin selection (Handberg and Stougaard 1992). Therefore, the hygromycin resistance gene was selected.

We developed four binary vectors supported with GATEWAY conversion technology. For overexpression of a transgene under the *Ljubq1* promoter, two binary destination vectors are available (Fig. 4). pUB-GW-GFP and pUB-GW-Hyg have the GATEWAY recombination site between the *Ljubq1* promoter plus the 5′ UTR intron and the *nos* terminator (Fig. 4). For gene silencing under the *Ljubq1* promoter, two binary destination vectors were constructed (Fig. 4): pUB-GWS-GFP and pUB-GWS-Hyg have tandem GATEWAY cassettes, in opposite orientation to each other. The intron1 of AtWRKY33 is located between the cassettes. These vectors are able to produce double-stranded RNA with the intron, whose structure leads to efficient gene silencing (Wesley et al. 2001). We also constructed pUB-GFP and pUB-Hyg for conventional cloning strategies without GATEWAY technology. The vectors shown in Figure 4 were deposited at the National BioResource Project Legume Base and are available upon request. The sequence of the genome clone LjT06D07 was deposited at the DNA Data Bank of Japan (DDBJ) under accession numbers AP009382 and AP009383. The full sequences of pUB-GW-GFP, pUB-GW-Hyg, pUB-GWS-GFP, pUB-GWS-Hyg, pUB-GFP, and pUB-Hyg were deposited at the DDBJ under accession numbers AB303064, AB303065, AB303066, AB303067, AB303068, and AB303069, respectively.

As a test, the *gus* reporter gene was cloned into pUB-GW-GFP and transformed into plants. The expected GUS staining was observed in nodules of transformed hairy roots (Fig. 5A). To test the efficiency of gene silencing, a partial sequence of the *gus* gene (approximately 450 bp) was cloned into pUB-GWS-GFP and the construct was transformed into the stably *gus*-transformed plants. Hairy root transformation often produces chimeric transgenic roots; therefore, we classified the transgenic roots with the pattern of GFP intensity, which is a transformation marker. In entirely strong GFP-expressed roots and in chimeric or weak GFP-expressed roots, 84 and 65% of roots were silenced, respectively (Table 1; Fig. 5B). This result suggests that the RNAi vectors are useful for gene silencing. In the case of no GFP expressed roots, 14% of roots were silenced (Table 1). This result may be due to the fact that the RNAi construct is transformed; however, the construct for GFP fails to be transformed in a certain frequency. Interestingly, we observed the GUS staining at 15% of the root tips even though
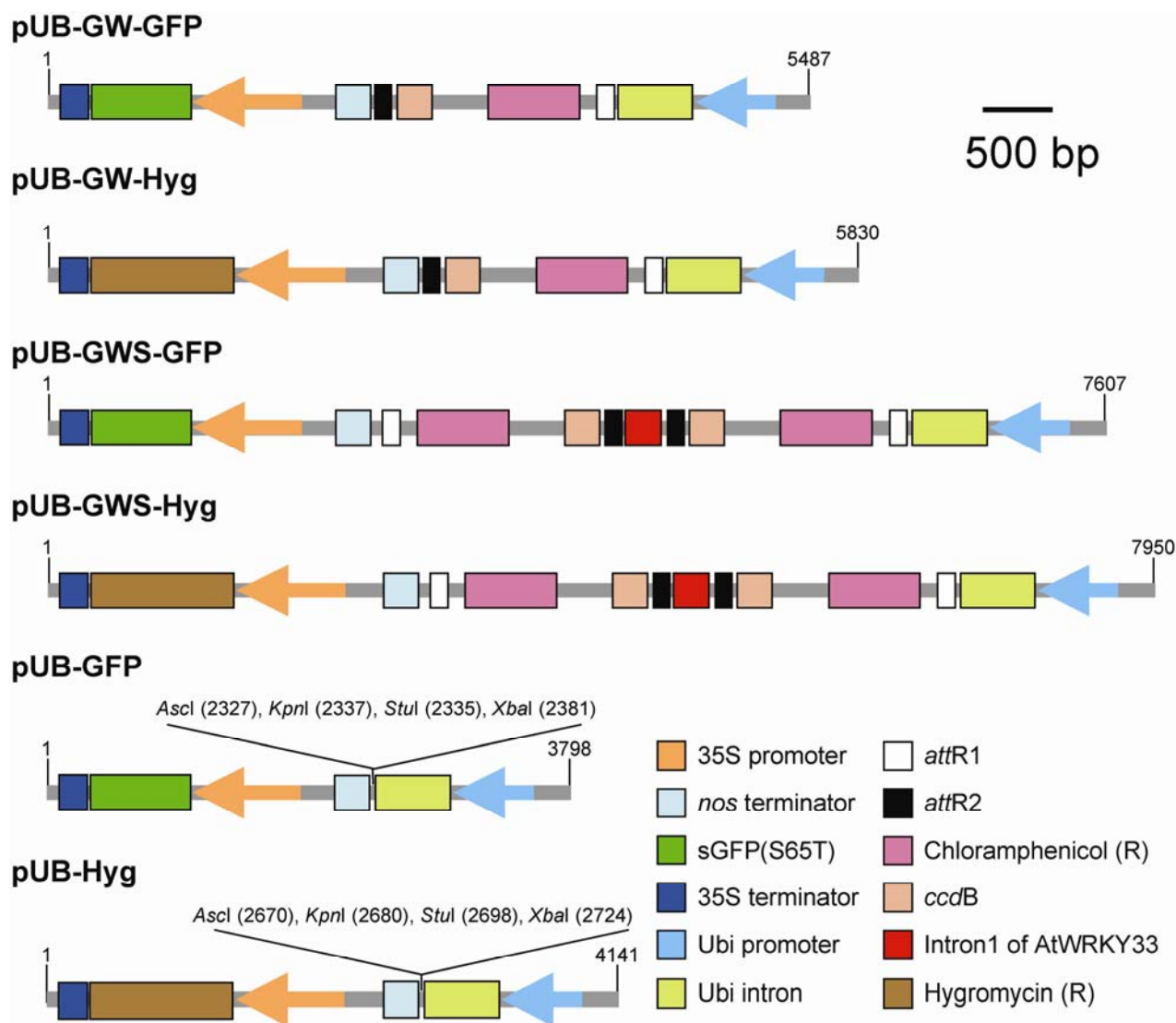


**Fig. 4.** Maps of *Ljubq1* promoter-based binary vectors. The binary vectors are represented as linear segments from the left to right borders. These constructs were based on a vector of which the 535-bp fragment of *Ljubq1* with the 5′ untranslated region intron that was cloned into pCAMBIA1300. The hygromycin resistance gene was originated from the backbone vector, pCAMBIA1300.

the other parts of the plants were silenced (Fig. 5C), indicating that root meristem is less susceptible to gene silencing. Similarly, gene silencing was not accomplished in nodule primordia, although other tissues were silenced (Fig. 5D). The mature nodules were silenced efficiently (Fig. 5E and F), except for a reminiscent GUS staining in nodule vasculatures (Fig. 5E, arrowhead).

The *Ljubq1* promoter-based binary vectors are now available for overexpression and gene silencing. The newly developed binary vectors are compatible with GATEWAY conversion technology that allows high-throughput cloning of interesting genes or DNA sequences. These vectors will help gene function analysis of *L. japonicus*.

## MATERIALS AND METHODS

### Transformation of *L. japonicus*.

*L. japonicus* accession Miyakojima MG-20 was used in this study. Hairy roots were induced using *Agrobacterium rhizogenes* LBA1334 (Offringa et al. 1986) or AR1193 (Stougaard et al. 1987) as described previously (Díaz et al. 2005). Stable transgenic plants were generated using *A. tumefaciens* LBA4404 (Jen and Chilton 1986) as described previously (Kato et al. 2005). Binary vectors were electroporated into bacteria using BTX Electro Cell Manipulator 600 (BTX Inc., San Diego, CA, U.S.A.) according to the manufacturer's instructions.

Transgenic plants were grown with nutrients premixed in Power Soil (Kureha Chemical Co., Tokyo) in a cultivation chamber (LPH-350S; Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan; and MLR-351H; Sanyo Electric Co., Ltd., Osaka, Japan) under a day-and-night cycle of 16 and 8 h and temperatures of 26 and 23°C, respectively.

### Rhizobial inoculation.

Surface-sterilized seed were germinated on 1% agar plate. The plates were covered with aluminum foil and set vertically for 2 days. The aluminum foil was removed and subsequently incubated for two more days under light in the cultivation chamber. The 4-day-old seedlings were transplanted to B&D nitrogen-free medium (Broughton and Dilworth 1971) with 1% Bacto agar (Becton Dickinson, Rutherford, NJ, U.S.A.) plate. After 3 days of incubation, *Mesorhizobium loti* MAFF303099 was inoculated. After 3 weeks of incubation, the leaves, stems, roots, and nodules were collected and used for GUS assays.

Plants with hairy roots were transplanted to B&D nitrogen-free medium with 1% bacto agar. After 3 days of incubation, *M. loti* MAFF303099 was inoculated. After 3 weeks of incubation, the roots and nodules were collected and used for GUS assays.

### GUS assays.

Histochemical GUS staining was performed as described previously (Stomp 1992) with minor modifications for leaf staining. Leaves were incubated in 90% acetone for 15 min at –20°C before staining. After 4 h of staining at 37°C, the samples were washed twice with 100 mM $NaPO_4$ (pH 7.0). The leaves were bleached with 70% ethanol at room temperature. Nodules were embedded with 6% agarose and the sections were prepared with a vibrating blade microtome (VT1000S; Leica Microsystems, Wetzlar, Germany). Mature pollen released from the anthers was stained for 2 h. The samples were observed with a light microscope (BX-50; Olympus, Tokyo) and photographed by a digital camera (DP70; Olympus). The activity of GUS in $T_1$ plants was measured with *p*-nitrophenol as described previously (Jefferson et al. 1986).

**Table 1.** Efficiency of gene silencing of β-glucuronidase (*gus*) by pUB-GWS-GFP[a]

| GFP intensity in hairy roots | Efficiency (%) (no. of roots unstained/total) |
|---|---|
| Entirely strong | 84 (16/19) |
| Chimeric or weak | 65 (11/17) |
| None | 14 (4/28) |

[a] GFP = green fluorescent protein. Hairy roots were stained for 4 h and unstained roots were counted as silenced roots.
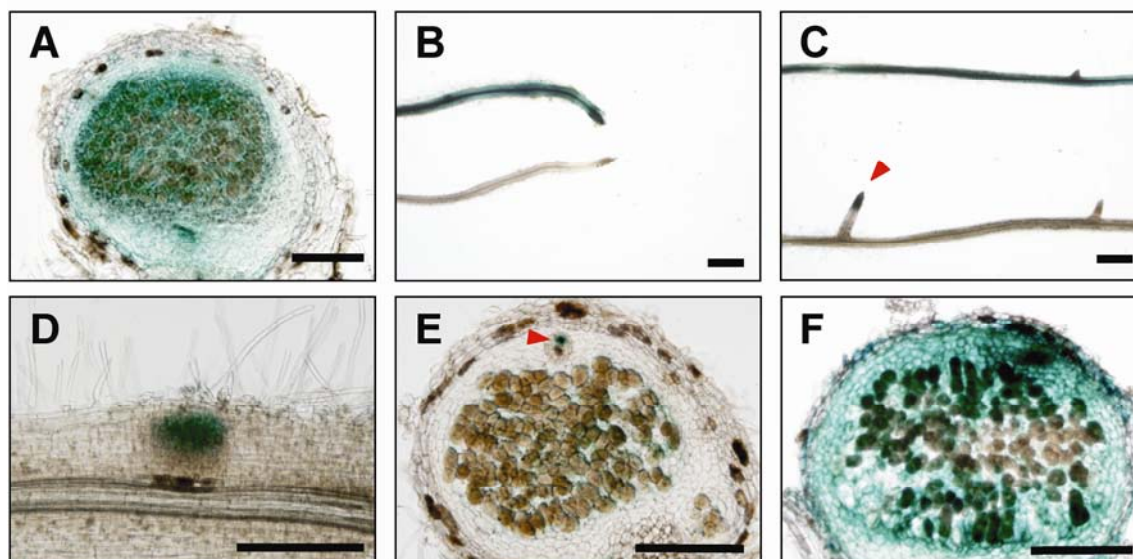


**Fig. 5.** Overexpression and gene silencing of β-glucuronidase (*gus*) transformed with *Ljubq1* promoter-based binary vectors in hairy roots. **A,** Overexpression of *gus* with pUB-GW-GFP. The *gus* of pBI121 was cloned into pUB-GW-GFP and transformed. **B through F,** Gene silencing of *gus* with pUB-GWS-GFP. Stably *gus*-transformed plants generated with pUB500i were used for the hairy root transformation. **B and C,** GFP-negative roots (upper) or positive roots (lower) were stained for 4 h. The pair of roots in each picture originated from the same shoot. **C,** Nonsilenced root tip was indicated by an arrowhead. **D,** A nodule primordium from the GFP-positive root. Mature nodules from the **E,** GFP-positive and **F,** GFP-negative roots. An arrowhead in E indicates a vascular bundle. Bars = **A, D** through **F,** 250 μm and **B** and **C,** 1 cm.

### In silico analysis.

The Gene Index Project of *L. japonicus* and cDNA Array Expression Profiles of *L. japonicus* (Kouchi et al. 2004) were used to identify the polyubiquitin gene, which potentially possessed the strongest transcriptional activity.

### Construction of binary vectors.

PCR was performed with KOD-Plus-DNA polymerase (Toyobo, Osaka, Japan) according to the manufacturer's instructions. The polymerase chain reaction (PCR) products or digested samples with restriction enzymes were purified with QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA ligation kit (version 2.1; Takara, Kyoto, Japan) was used for DNA ligation according to the manufacturer's instructions. For the construction of pUB500, pUB1100, pUB500i, and pUB1100i, the CaMV35S promoter of pBI121 (Jefferson et al. 1987) was replaced by fragments of *L. japonicus* ubiquitin (*Ljubq1*) as follows. First, the regions of the *Ljubq1* promoter and the intron were amplified from a *L. japonicus* genomic clone, LjT06D07, with the primers below: ub500, 5′-GGAGAGAGGATTTTGAGGAA-3′ and ub-X, 5′-CTAGTCTAGAGAATTGTTAGGGTTGGGAAC-3′ for pUB500; ub1100, 5′-CTTCCATACTTTCATGGTGTG-3′ and ub-X for pUB1100; ub500 and ub-intron-X, 5′-CTAGTCT AGACTGTAATCACATCAACAACAGA-3′ for pUB500i; and ub1100 and ub-intron-X for pUB1100i (Fig. 1). The fragments were digested with *Xba*I. pBI121 was digested with *Hin*dIII and treated with Klenow fragment (Takara) to create blunt ends. Then the treated pBI121 was digested with *Xba*I and ligated into each fragment of *Ljubq1*.

To construct binary destination vectors supported with GATE-WAY conversion technology (Invitrogen, Carlsbad, CA, U.S.A.) as shown in Figure 4, pCAMBIA1300 (Cambia) was used as a backbone in the general structure of *Ljubq1* promoter-based binary vectors. As a selectable marker for plant transformation, we used the hygromycin-selectable marker gene of pCAMBIA1300 or sGFP (S65T) gene of modified pHKN29,pHKN29r (Kumagai and Kouchi 2003; Niwa et al. 1999). As by-products, we also constructed pUB-GFP and pUB-Hyg for conventional cloning strategies without GATEWAY technology.

The open reading frame of the *gus* gene on pBI121 was amplified with GUScds-attB1, AAAAAGCAGGCT<u>ATGTTAC GTCCTGTAGAAAC</u> and GUScds-attB2, AGAAAGCTGGGT <u>TCATTGTTTGCCTCCCTGCT</u> (the underlines indicate specific sequences of the *gus* gene). The PCR products were reamplified with attB1 adapter, 5′-GGGGACAAGTTTGTACAAA AAAGCAGGCT-3′ and *att*B2 adapter, 5′-GGGGACCACTTT GTACAAGAAAGCTGGGT-3′. The *att*B recombination sites flanking the PCR product were subcloned into pDONR/Zeo (Invitrogen) using BP clonase II enzyme mix (Invitrogen) according to the manufacturer's instructions. The *gus* sequence on the pDONR/Zeo subsequently was transferred into pUB-GW-GFP using LR clonase II enzyme mix (Invitrogen) according to the manufacturer's instructions.

The partial sequence of the *gus* gene on pBI121 (445 bp) was amplified with GUS-attB1, 5′-AAAAAGCAGGCT<u>GGGC CAACAGTTCCTGATTA</u>-3′ and GUS-attB2, 5′-AGAAAGCT GGGT<u>CGAAATATTCCCGTGCACTT</u>-3′ (the underlines indicate specific sequences of the *gus* gene). The PCR product was reamplified with attB1 adapter and attB2 adapter. The partial sequence of the *gus* gene was transferred into pUB-GWS-GFP.

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## AUTHOR-RECOMMENDED INTERNET RESOURCES

National BioResource Project (NBRP) Legume Base:
www.legumebase.agr.miyazaki-u.ac.jp/

The Gene Index Project: compbio.dfci.harvard.edu/tgi/

Kazusa DNA Research Institute cDNA Array Expression Profile website:
www.kazusa.or.jp/en/plant/lotus/EST/cDNA.html