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Original Paper

A MYB-related transcription factor affects nodule formation in *Lotus japonicus*

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Abstract The MYB family is one of the largest groups of transcription factors in plants. Our previous microarray data revealed that a MYB-related gene (*Ljmybr*) was induced upon nodule formation in *Lotus japonicus*. As shown using real-time PCR, the expression of the *Ljmybr* in *L. japonicus* was up-regulated after *M. loti* infection. To determine the role of *Ljmybr* in the nodulation process, we created transgenic hairy roots in *L. japonicus* and then performed *Ljmybr* overexpression (MYBOX) or RNAi-mediated suppression (MYBRNAi). The results of these experiments indicated that overexpressing *Ljmybr* accelerated *L. japonicus* nodulation and increased acetylene reduction activity (ARA) in the nodules. Suppressing *Ljmybr* (MYBRNAi) delayed nodulation and decreased ARA compared to control suppression (GUSRNAi). Seven days post infection, the expression levels of *Nin*, *Enod40-1* and *Enod40-2* were higher in MYBOX-treated nodules and lower in MYBRNAi-treated nodules. We propose that the *Ljmybr* transcription factor plays a role in regulating nitrogen fixation in *L. japonicus*.

Key words: *Lotus japonicus*, Myb-related transcription factor, nodule, overexpression, RNAi.

Leguminous plants are capable of establishing nitrogen fixing symbiosis with soil bacteria of the family Rhizobiaceae. Under nitrogen deficiency conditions, soil bacteria (rhizobia) infect the roots of legumes and induce the formation of root nodules, while host plants supply organic acids to the bacteria in exchange for ammonia. This critically important symbiotic relationship provides approximately 40 million tons of nitrogen to agricultural systems each year (Peoples et al. 2009).

During the symbiosis between rhizobia and leguminous plants, symbiotic interactions play a crucial role (Ferguson and Mathesius 2003). Through recent molecular studies, important progress has been made towards understanding the initial stages of this complex developmental process. Nod factors, the signaling molecules carried by rhizobia, can activate a signaling pathway resulting in transcriptional activation. The regulatory proteins responsible for this activation bind to specific sequences of promoter regions, which control the activity of genes in response to environmental stresses. In legumes, several transcription factors have been identified by their capacity to regulate the nodulation process (Rípodas et al. 2014).

Previously, researchers performed transcriptome or RNA-seq analysis and identified many upregulated genes in *Lotus japonicus* nodules after *Mesorhizobium loti* infection (Chungopast et al. 2014; Handa et al. 2015; Kouchi et al. 2004). From these data, we found one upregulated gene, MYB-related transcription factor (*Ljmybr*). The MYB transcription factors have been reported to be composed of one to three DNA-binding domains containing more than 50 amino acids (Rosinski and Atchley 1998). The MYB proteins are classified into three major subfamilies (R2R3-MYB, R1R2R3-MYB and MYB related proteins, which contain a single MYB repeat) depending on the number of adjacent sequences that repeat in the MYB domain. In plants, MYB transcription factors participate in cell development, environmental response and hormone regulation (Stracke et al. 2001).

In *L. japonicus*, some MYB transcription factor functions have been reported. Volpe et al. (2013) found that MYB transcription factors were upregulated in *L. japonicus* roots during mycorrhizal infection. Kang et al. (2014) reported that the MYB subfamily member IPN2 is important for nodule organogenesis. The RNAi-mediated suppression of IPN2 significantly decreased the number of nodules in *L. japonicus*. In this report, we examine

Abbreviations: ARA, acetylene reduction activity; dai, days post infection.
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one of the MYB-related transcription factors, which is induced by *M. loti* infection in root tissue.

Materials and methods

Plant growth and M. loti infection

L. japonicus B-129 (GIFU) seeds were surface-sterilized and germinated on sterile vermiculite with 1/2 liquid B&D medium (Broughton and Dilworth, 1971) in double Magenta jars. The plants were grown in a growth cabinet (EYELA FLI-2000, Japan) at 24°C with 16 h / light and 8 h / dark. After one week, plants were inoculated with 10 ml of *M. loti* MAFF303099. *M. loti* has been incubated in TY Medium (Beringer 1974) for 3 days. Then, the cell density was adjusted to 1×10^9 cells ml^{-1}.

Construction of MYBRNAi and MYBOX in L. japonicus

For *Ljmybr* (chr4.CM0004.2240.r2.d) suppression using RNAi, a 289-bp fragment (position 544- 832) was amplified from cDNA using the following primers, which contain attB recombination sites for use with the Gateway vector: FW primer; 5′-ACA AGT TTG TAC AAA AAG CAG GCT GTT GGG AAT GAT GCC ACT TT-3′; Rv primer; 5′-ACC ACT TTG TAC AAG AAA GCT GGG TAC GGT AAT TGG TCC AAG CAG-3′. For Myb overexpression (MYBOX), a 900-bp fragment was amplified using the following primers: FW primer; 5′-ACA AGT TTG TAC AAA AAG CAG GCT ATG GGC AGG AAG TGC TCA CA-3′; Rv primer; 5′-ACC ACT TTG TAC AAG AAA GCT GGG TTT AAG TCA CGG TAA TTG GTC-3′ (the underline indicates the attB recombination sites). The PCR products were subcloned into pDNOR/Zeo (Invitrogen) and then transferred into a Gateway binary vector, pUB-GWS-GFP, for RNAi and pUB-GW-GFP for *Ljmybr* overexpression (Maekawa et al. 2008). To construct GUSRNAi or GUSOX, pENTR-gus was digested with *Nhe*I. The resulting GUS fragment was cloned into pUB-GWS-GFP as a suppression control (GUSRNAi) or pUB-GW-GFP as an overexpression control (GUSOX).

Hairy root transformation

Hairy root transformation of *L. japonicus* Gifu using *Agrobacterium rhizogenes* LBA1334 was performed according to a previously described procedure (Kumagai and Kouchi 2003). Seedlings (5–6 days old) were placed in a petri dish containing an *A. rhizogenes* suspension and then cut at the base of the hypocotyl. For co-cultivation, the treated seedlings were transferred onto agar plates containing 1/2 strength Jensen medium (Díaz et al. 1989), placed vertically in a growth cabinet and grown for 5 days. Then, the plants were transferred onto HRE agar medium containing 150 mg ml^{-1} cefotaxime and grown for 10 more days. Hairy roots that emerged from the base of the hypocotyls were tested for GFP fluorescence. The plants with transgenic hairy roots were transferred to vermiculite supplied with 1/2 strength B&D medium. The transformed plants were inoculated with *M. loti* and grown in a growth cabinet. Nodules were sampled at each stage.

Histochemical analysis of Ljmybr promoter-fused GUS in L. japonicus

A 1.0-kb fragment upstream of the start codon of the *Ljmybr* transcription factor was amplified from genomic DNA of *L. japonicus* using PrimeSTAR HS DNA polymerase (Takara, Shiga, Japan) with the following specific primers: forward primer, 5′ AAA TCT AGA GAG ACT ATT GTT TTG GTC AG-3′; reverse primer, 5′-TTT CCC GGG TTT CAC TTG AAC AAG ATT TT-3′. The PCR fragment was digested with *Xba*I and *Sma*I. The resulting fragment was ligated into pCAMBIA fused to the GUS gene (Mai et al. 2006). The construct was then introduced into *L. japonicus* using hairy root transformation and GUS staining (Mai et al. 2006) using 5-bromo-4-chloro-3-indolyl-β-D-glucuronide cyclohexylammonium salt (X-gluc) was performed.

Acetylene reduction activity

To analyze the nitrogen fixing activity, the acetylene reduction activity (ARA) was performed using Shimadzu GC-8A gas-chromatography (Shimazu, Kyoto, Japan) (Banba et al. 2001). Ethylene was used as a standard. To measure the ARA, nodules were detached from each transgenic hairy root, placed into a 25-ml vial and incubated at 37°C with 2.6 ml of acetylene. After 30 min, ethylene formation was measured by gas chromatography.

Quantitative real time PCR

The total RNA from roots or nodules was extracted using a RNeasy plant mini kit (Qiagen, CA, USA). Reverse transcription was performed using the PrimeScript RT master mix (Takara, Shiga, Japan), and then, quantitative real-time PCR was performed (95°C for 30 s, 40 cycles of 95°C for 5 s and 60°C for 30 s) using Takara SYBR Green premix ExTaqII (Takara, Shiga, Japan) with a set of primers specific to *Ljmybr:* forward; 5′-AGC ACC TGA CCT GGA GCT TA-3′ and reverse; 5′-ATT GGT CCA AGC AGG AAT TG-3′. The following primers were used to amplify Leghemoglobin: forward; 5′-AGT TGG GGA CAA ATG GAG TG-3′ and reverse; 5′-CAT AGG CTA CTC CCC AAG CA-3′. Ubiquitin was used as internal control for relative quantification (forward; 5′-ATG CAG ATC TTC GTC AAG ACC TTG-3′, reverse, 5′-ACC TCC CCT CAG ACG AAG-3′).

Primers for the Enod40-1, Enod40-2, and Nin primers were described previously (Kumagai et al. 2006). The Ljmate1 primers were described by Takanashi et al. (2013).

Results

Ljmybr expression analysis using quantitative real-time PCR and histochemical localization using GUS staining

Many researchers have performed transcriptome or RNA-seq analyses in *L. japonicus* and identified genes

upregulated after *M. loti* infection (Chungopast et al. 2014; Handa et al. 2015; Kouchi et al. 2004). From these data, we found that the *Ljmybr* transcription factor (chr4.CM0004.2240.r2.d) was up-regulated in roots after *M. loti* infection. To determine the expression of the *Ljmybr* transcription factor during nodulation, we investigated the transcript levels of *Ljmybr* at each developmental stage of roots or nodules (Figure 1). The relative transcript levels compared to ubiquitin were calculated. *Ljmybr* expression was induced in the early stages, beginning 3 days after infection (dai); reached maximum levels at 14 and 28 dai; and decreased at 56 dai (Figure 1A). The expression of nodule inception (Nin) and several early nodulin genes (Enod40-1, Enod40-2) express at 3 dai, the early stage of *M. loti* infection (Figure 1B-D). Both the citrate transporter (Ljmate1) and leghemoglobin (Lb) are markers for nitrogen fixation (Figure 1E, F). These data reveal that following *M. loti* infection, the *Ljmybr* gene is expressed from the early stages of nodule development until the nodules senesce.

To determine the localization of *Ljmybr* gene expression, we generated a construct fusing the *Ljmybr* promoter to the GUS gene. GUS staining was observed at the nodule bumps and vascular bundles of the root (Figure 2A). When nodules became mature, only the vascular bundles were stained in the nodules and root (Figure 2B).

Phenotypes following Ljmybr overexpression or suppression in transgenic hairy roots inoculated with M. loti

We generated *Ljmybr* overexpression (MYBOX) and suppression (MYBRNAi) constructs in transgenic hairy roots. GUS overexpression (GUSOX) and suppression (GUSRNAi) constructs were used as controls. These constructs contained green fluorescence protein (GFP) as a positive marker for transgenic roots. We measured 44 independent transgenic hairy roots. Figure 3 shows the phenotypes of transgenic hairy roots inoculated with *M. loti* 14 dai. The number of nodules in MYBOX-treated roots was higher than the number of nodules in GUSOX -treated roots (Figure 3A, B). In contrast, the MYBRNAi-treated roots carried a lower number of nodules than GUS RNAi-treated roots. Although the number of nodules in MYBOX-treated roots was higher compared to GUSOX-treated roots at 14 dai, the

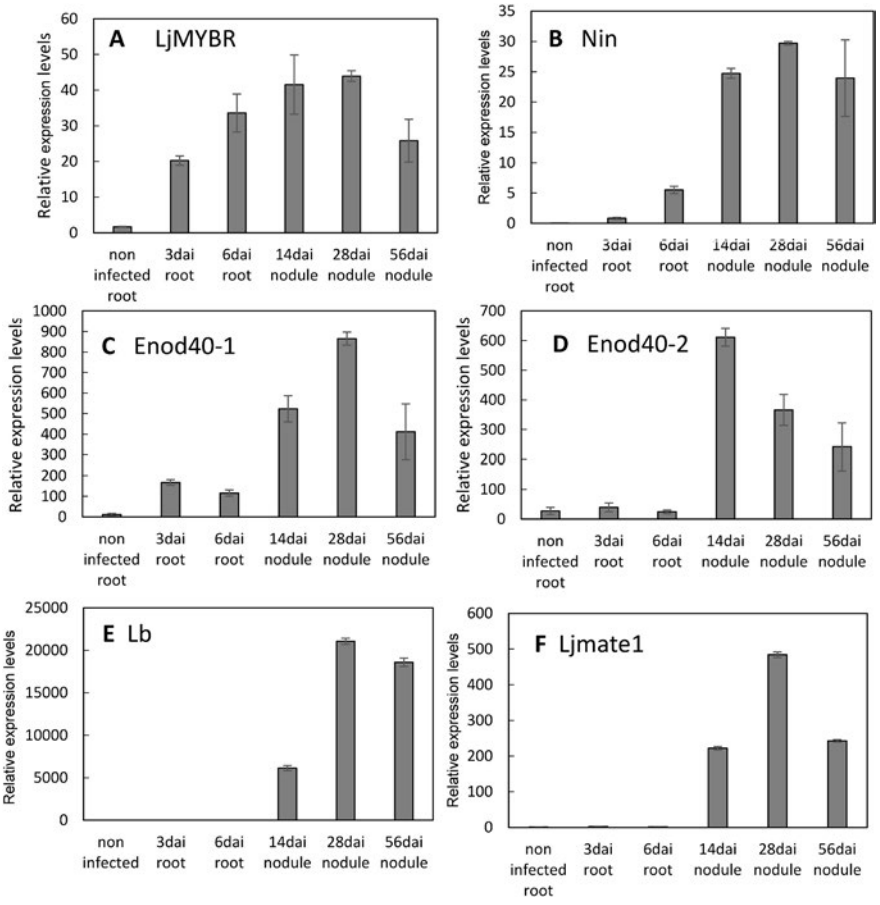

Figure 1. Expression of various genes during nodule development in *Lotus japonicus*. Total RNA was isolated from roots or nodules at various days after infection (dai). Transcript levels were estimated by quantitative real-time RT-PCR. The expression levels were normalized to those of ubiquitin. The error bars indicate the mean±*SD*.

numbers of nodules were similar at 28 dai (Figure 4A). In contrast, the number of nodules at 14 dai in MYBRNAi-treated roots was lower compared to GUSRNAi-treated roots (Figure 4B). We measured the acetylene reduction activity (ARA) in the nodules to estimate their nitrogen fixing capability (Figure 4C, D). The ARA of nodules in MYBOX-treated roots was higher compared to that of GUSOX-treated roots, and the ARA of nodules in the MYBRNAi-treated root was slightly lower than that in GUSRNAi-treated root. These data revealed that overexpression of the *Ljmybr* transcription factor accelerated nodulation and induced increased ARA until senescence. On the other hand, the suppression of the *Ljmybr* transcription factor delayed nodulation and suppressed ARA, suggesting that LjMYBR might regulate nitrogen fixation.

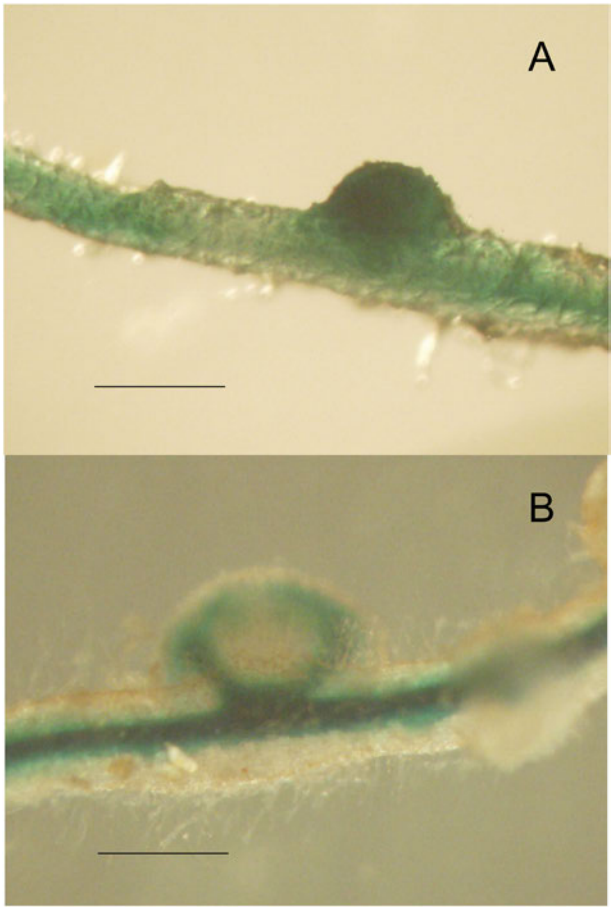

Figure 2. Expression analysis of *Ljmybr*. GUS staining of *Ljmybr* promoter::GUS transformants. Whole nodules were stained with X-gluc. Scale bar; 0.5 mm. A, nodule bump at 10 dai; B, mature nodule at 14 dai.

Expression of various genes in transgenic hairy roots

Our data showed that overexpression of LjMYBR accelerated nodulation and suppression of LjMYBR delayed nodulation, suggesting that the LjMYBR transcription factor might play a role as a transcriptional regulator of nodulation. To confirm this speculation, we measured the transcript levels of various genes in each transgenic nodule by quantitative real-time PCR (Figure 5). The relative transcript level of each gene compared to ubiquitin was calculated. Total RNA was isolated from the roots at 7 dai (Figure 5A-D). Subsequently, cDNA was synthesized from RNA and measured by quantitative real-time PCR using gene-specific primers. The expression of *Ljmybr* in MYBOX-treated roots was 7 times higher than in GUSOX-treated roots. The expression of *Ljmybr* in MYBRNAi-treated roots was lower, but not significantly so (Figure 5A). The expression levels of *Nin*, *Enod40-1* and *Enod40-2* in MYBOX-treated roots were higher than in GUSOX-treated roots, and the expression levels of these genes in MYBRNAi-treated roots were lower than in GUSRNAi-treated roots (Figure 5B-D). Then, we measured the expression of *Ljmybr* in the nodules of transgenic hairy

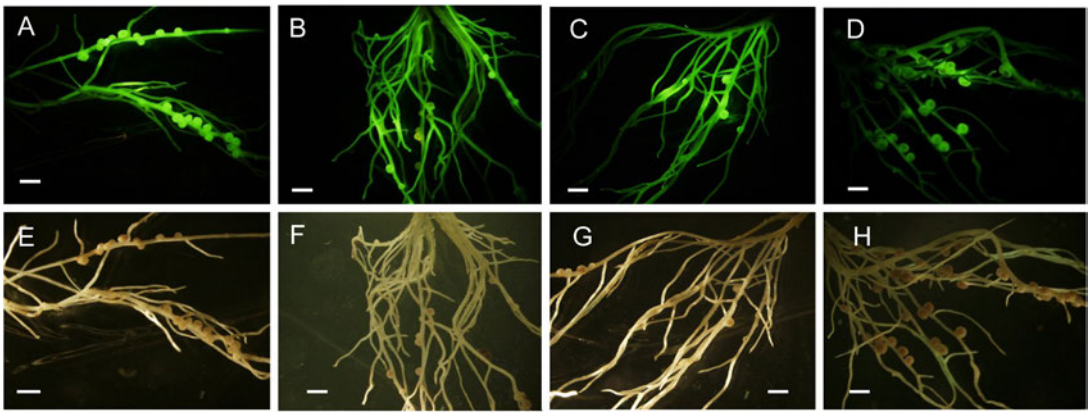

Figure 3. Phenotypes of *Ljmybr* overexpression (MYBOX) and RNAi-mediated suppression (MYBRNAi) in transgenic hairy roots. Roots were transformed with MYBOX and MYBRNAi, or control vectors, GUSOX and GUSRNAi, respectively, and then inoculated with *M. loti* for 14 days. GFP was used as transformation marker. Hairy roots were observed by fluorescence (A-D) and bright field (E-H) microscopy. MYBOX (A, E), GUSOX (B, F), MYBRNAi (C, G), GUSRNAi (D, H). Scale bar; 2 mm.

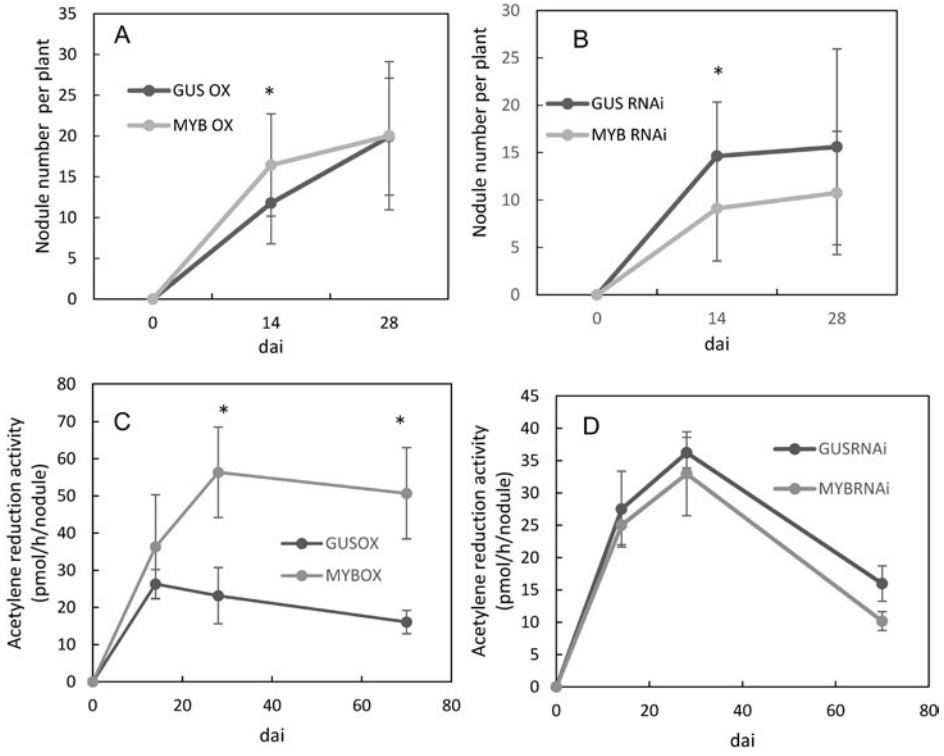

Figure 4. Nodule number and acetylene reduction activity in transgenic hairy roots. The nodule numbers of overexpression (A) and suppression (B) construct-treated roots. Acetylene reduction activities in overexpression (C) and suppression (D) construct-treated roots. Statistical analysis was performed using Tukey's test for multiple comparisons. Significant differences were determined by $p<0.05$. $n>35$.

roots at 28 dai (Figure 5E-H). The expression of *Ljmybr* in MYBOX-treated nodules was approximately 2 times higher than in GUSOX-treated nodules, and the expression of *Ljmybr* in MYBRNAi-treated nodules was approximately 4 times lower than GUSRNAi-treated nodules (Figure 5E). However, the expression levels of *Nin*, *Enod4-1* and *Enod40-2* in MYBOX and MYBRNAi-treated nodules were similar to the expression levels of the control GUSOX and GUSRNAi-treated nodules, respectively. These data suggest that the LjMYBR transcription factor might play a role in nodulation activation, although this transcription factor might have other functions in nitrogen fixing regulation in the nodule.

Discussion

Plant MYB transcription factors are classified into three to four groups (R2R3- R3- or 1R- and MYB-related families) that contain one to three conserved MYB DNA binding domains (a helix-turn-helix (HTH) domain) with more than 50 amino acids (Du et al. 2013). In this study, we focused on one of the MYB-related genes, *Ljmybr*, which is expressed after *M. loti* infection. Although there are many reports about R2R3-MYB proteins, little is known about MYB-related genes (Du et al. 2012a; Du et al. 2012b; Matus et al. 2008; Stracke et al. 2001). In maize and soybean, MYB-related genes have roles both in development and in stress responses (Du et al. 2013). Based on the alignment of the MYB domain, MYB-related proteins have been classified into at least five groups (Du et al. 2013). The third helix in the MYB DNA binding domain is important for binding to target cis elements (Du et al. 2013). The CCA1 (circadian clock associated 1)-like subgroup of the MYB-related proteins contains a highly conserved SHAQK(Y/F)F motif in the third helix of the MYB domain. LjMYBR has an identical domain, SHAQKY. A BLAST search showed that LjMYBR is highly conserved to At5g56840 or *GmMYBR047a* in *Arabidopsis* or soybean. Interestingly, *GmMYBR047a* is expressed at higher levels in nodules compare to other organs (Du et al. 2013). These genes are members of the MYB-related CCA1 group. CCA1-like MYB proteins are known to play roles in the regulation of circadian rhythm and flower development in *Arabidopsis*, maize and soybean (Fujiwara et al. 2008; Schaffer et al. 1998). Recently, the CCA1 subgroup is divided into four major clades according to intron patterns (Du et al. 2013). The clades that contain genes that regulate circadian rhythmicity (clade III or VI) are different from the clade containing LjMYBR, which according to its alignment with At5g56840 or *GmMYBR047a* belongs to clade I (Du et al. 2013). The MYB-related genes in clade I regulate seed development and germination as well as environmental stress responses, such as drought or cold stress (Churin et al. 2003; Rubio-Somoza et al. 2006; Shin

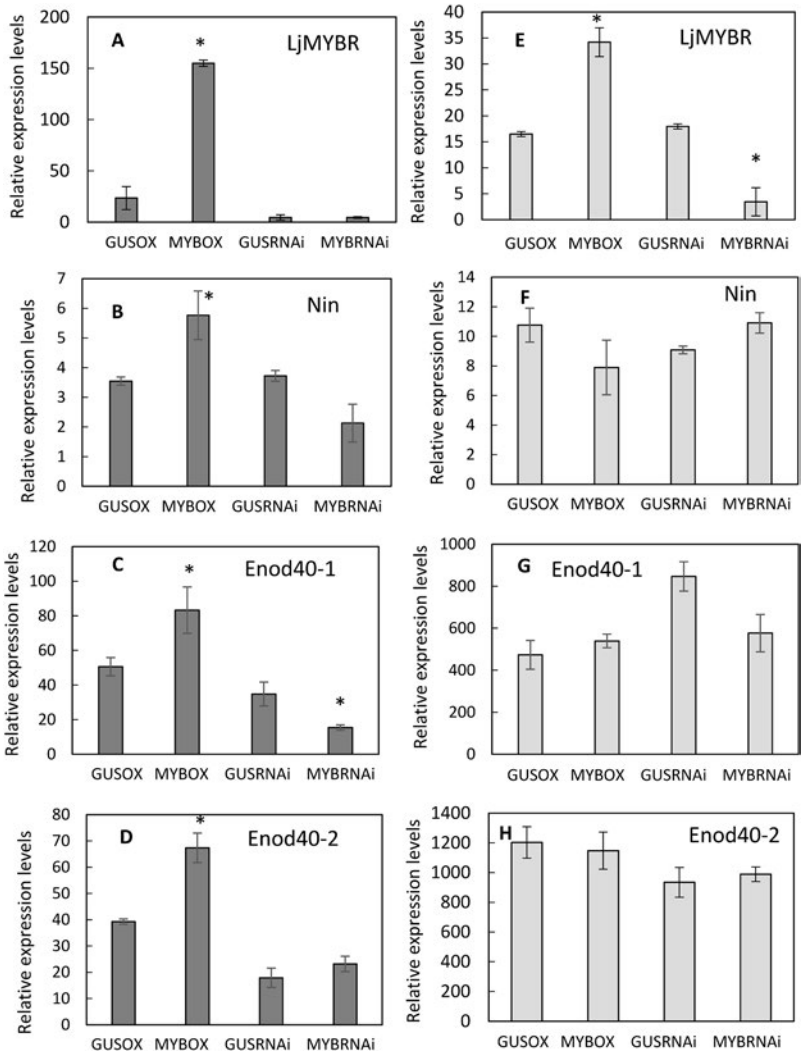

Figure 5. Expression of various genes of transgenic hairy roots in *L. japonicus*. Total RNA was isolated from GFP positive roots at 7 dai (A-D, dark gray) or GFP positive nodules at 28 dai (E-H, light gray). Transcript levels were estimated by quantitative real time RT-PCR. The expression levels were normalized to ubiquitin. The error bars indicate the mean±*SD*. $p<0.01$.

et al. 2011; Su et al. 2010). Because nodule nitrogenase activity is sensitive to the oxygen concentration (Weisz and Sinclair 1987), LjMYBR might contribute to the maintenance of a sufficiently low oxygen environment in nodules. Although the ARA in MYBOX-treated nodules was significantly higher than in GUSOX-treated nodules, the ARA in MYBRNAi-treated nodules was slightly lower compared to that of GUSRNAi-treated nodules (Figure 4). We do not know why the ARA in MYBRNAi-treated nodules was not different from that of GUSRNAi-treated nodules. This may be because the suppression of *Ljmybr* in MYBRNAi-treated nodules was not sufficient. The expression of *Lb* or *Ljmate1* in MYBOX- or MYBRNAi-treated nodule was similar to control nodules (GUSOX or GUSRNAi, data not shown). These data indicate that LjMYBR regulates the stress-inducible genes necessary to maintain low oxygen conditions in the nodule.

Enod40, as an early nodulin gene, has been investigated in some studies as one of the genes responsible for controlling nodule development (Fang and Hirsch 1998). The overexpression of ENOD40 accelerated nodulation. On the other hand, the knock down of ENOD40 suppressed nodule formation (Charon et al. 1999; Kumagai et al. 2006; Wan et al. 2007). The expression pattern of *Ljmybr*, *Enod40* and *Nin* are similar (Figure 1). *Ljmybr*- or *Enod40*-promoter induced GUS expression in the vascular bundles of the nodule (Figure 2, Martirani et al. 1999). *Nin* is expressed in the nodule primordia and in the central tissue and vascular bundles of mature nodules (Schauser et al. 1999). These results indicate that not only Nin but also LjMYBR might regulate *Enod40* expression. LjMYBR might play a role in the repression or the post-transcriptional regulation of *Enod40*. In *Arabidopsis*, two transcriptional repressor domains have been conserved (Ikeda and Ohme-Takagi 2009). One of these domains is an ethylene responsive element binding factor-associated amphiphilic repression (EAR) motif with a LXLXL amino acid sequence. The

other domain is a L/VR/KLFGVXM/V/L motif, and the RLFGV sequence acts as a repressor (Ikeda and Ohme-Takagi 2009). In *Arabidopsis*, overexpression of At2g3680, which has the LRLFGVNM domain, caused the loss of the shoot apical meristem (SAM) and resulted in the production of narrow leaves and bushy rosettes. The deletion mutant displayed a normal phenotype (Ikeda and Ohme-Takagi 2009). LjMYBR has both conserved LXLXL and LRLFGVQL amino acid sequences, indicating that the overexpression and suppression of LjMYBR might play a dominant repressor role in the transgenic nodules. However, we do not know which genes are regulated by the LjMYBR transcription factor. Further study is necessary to determine the function of LjMYBR in *L. japonicus*.

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