

Isolation and molecular characterization of a *Lotus japonicus* R2R3-MYB subgroup 7 transcription factor gene

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Abstract We isolated an ortholog (*LjMYB12*) of the Arabidopsis R2R3-MYB transcription factor (TF) gene from *Lotus japonicus* to investigate the regulation of flavonoid biosynthesis, which is driven by many paralogous genes in *L. japonicus*. We characterized the spatial and temporal expression of *LjMYB12* in leaves, stems, roots, flowers, immature seeds, seedling leaves, and seedling roots. Expression was much higher in flowers than in other tissues. To verify the relationship between the expression of *LjMYB12* and that of flavonoid biosynthesis genes, we generated transgenic *L. japonicus* plants overexpressing *LjMYB12*. Overexpression of *LjMYB12* resulted in the upregulation of genes for a chalcone synthase paralog (*CHS1*), flavanone 3-hydroxylase, and flavonol synthase. Interestingly, *LjMYB12* strongly activated *CHS1* but did not activate other *CHS* paralogs. This result suggests differences in the spatial or temporal activation of *CHS* paralogs by R2R3-MYB TFs. Molecular characterization of R2R3-MYB TFs in *L. japonicus* will reveal the effects of gene duplication on the regulation of diverse flavonoid biosynthesis.

Key words: chalcone synthase, flavonol synthase, flavanone 3-hydroxylase, spatial and temporal expression, transgenic *Lotus*.

Leguminous plants use flavonoids and their derivatives in their interactions with other organisms and in response to various environmental stresses (Aoki et al. 2000; Denarie et al. 1992). The regulation of flavonoid biosynthesis in legumes is more complicated than that in other higher plants, because multiple paralogous genes involved in flavonoid biosynthesis exist in the genomes of legumes (Clough et al. 2004; Ryder et al. 1987; Shimada et al. 2003). Spatial and temporal expression of these paralogous genes and the functions of their products are highly diverse (Shimada et al. 2003, 2005). In *Arabidopsis thaliana*, the expression of flavonoid biosynthesis genes is regulated by several R2R3-MYB transcription factors (TFs) (Borevitz et al. 2000; Nesi et al. 2001). The modular structure of these proteins, which consist of the MYB DNA-binding domain in the N-terminal region and the activation or repression domains in the C-terminal region, places them in one of phylogenetic subgroups 4 to 7 (Kranz et al. 1998; Stracke et al. 2001). In addition, orthologs in subgroups 4 to 7 regulate the expression of flavonoid biosynthesis genes in several other species (Czemmel et al. 2009; Lin-Wang et al. 2010; Paz-Ares et al. 1987). Among R2R3-MYB subgroup 4 to 7, Arabidopsis AtMYB12, which is classified into subgroup 7, is well characterized on the flavonoid biosynthesis (Lewis

et al. 2011; Mehrtens et al. 2005; Stracke et al. 2010). Homologs of AtMYB12 also regulated the expression of genes related to flavonoid biosynthesis in tomato (*Solanum lycopersicum*) and grapevine (*Vitis vinifera*) (Ballester et al. 2010; Czemmel et al. 2009). Therefore, the molecular characterization of R2R3-MYB subgroup 7 in legumes would reveal the mechanisms that control the expression of many paralogous genes related to flavonoid biosynthesis. In this study, we isolated an ortholog of R2R3-MYB subgroup 7 from *Lotus japonicus*, a model plant for the study of the molecular genetics of legumes, on the basis of sequence similarity. To understand the regulation of the flavonoid biosynthesis pathways in *L. japonicus*, we compared spatial and temporal expression patterns between the isolated R2R3-MYB subgroup 7 gene and flavonoid biosynthesis genes. We generated transgenic *L. japonicus* plants overexpressing the isolated R2R3-MYB subgroup 7 gene and evaluated the expression of flavonoid biosynthesis genes in these plants.

L. japonicus accession Gifu B-129 was obtained from Biological Resource Center in *Lotus japonicus* and *Glycine max* of the National BioResource Project (University of Miyazaki). Plants were grown in commercial soil at 26°C in a 16-h light (100–150 μmol s⁻¹ m⁻²)/8-h dark regime in a growth chamber.

Three R2R3-MYB subgroup 7 proteins in Arabidopsis (AtMYB11, AtMYB12, and AtMYB111) contain the R2R3-MYB DNA-binding domain in the N-terminal region and the consensus motif 'GRTxRSxMK' (Kranz et al. 1998). The motif of 'GRTxRSxMK' serves as a characteristic for distinguishing R2R3-MYB subgroup 7 from the other subgroups. In silico EST sequence analysis was used to search EST clones corresponding to R2R3-MYB subgroup 7 in *L. japonicus*. We found one EST clone (CN824947) with the motif conserved in R2R3-MYB subgroup 7 among EST clones of *L. japonicus*. Since the sequence information of the CN824947 clone was partially, the full-length cDNA was synthesized from *L. japonicus*. Total RNA was extracted from plants with Trizol reagent (Life Technologies, USA). First-strand cDNA was synthesized from total RNA (~1 µg) with oligo(dT) primer and ReverTra Ace reverse transcriptase (Toyobo, Japan). The full-length cDNA for the R2R3-MYB subgroup 7 gene was synthesized using a 3'-Full RACE Core Set (TaKaRa, Japan) and one specific primer designed on the basis of the sequence information of

CN824947 (Supplemental Table). We evaluated the expression levels of *LjMYB12*, 4-coumarate: CoA ligase (4CL), cinnamic acid 4-hydroxylase (C4H), 3 chalcone isomerases (CH11-3), 5 chalcone synthases (CHS1, 2, 3, -L1, and -L2), flavanone 3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H), 2 isoflavone synthases (IFS1, 2), isoflavone reductase (IFR1), flavonol synthase (FLS), and dihydroflavonol 4-reductase (DFR) by qRT-PCR using SYBR Premix ExTaq II (Tli RNaseH Plus; TaKaRa, Japan) and the primers listed in the Supplemental Table. The reaction was performed in a CFX96 Real-Time System (Bio-Rad Laboratories Inc., Japan) under the following conditions: 40 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 30 s. The plasmid vectors pMDC100-IG and pMDC100-*LjMYB12* (Supplemental Figure 1) were constructed by joining the *intron-GUS* reporter gene (Ohta et al. 1990) or the *LjMYB12* gene, respectively, to the cauliflower mosaic virus 35S promoter in the original pMDC100 vector (Curtis and Grossniklaus 2003). Gifu B-129 was transformed and plants were regenerated as described by Stiller et al. (1997) with modifications:

AtMYB11	1:	MGRAPCCEKVGIIKGRWTAEDRTLSDYIQSNGEGSWRSLPKNAGLKRCGKSCRLRWINY	: 60
AtMYB12	1:	MGRAPCCEKVGIIKGRWTAEDQILSNYIQSNGEGSWRSLPKNAGLKRCGKSCRLRWINY	: 60
AtMYB111	1:	MGRAPCCEKIGLKRGRWTAEEDEILTKEYIQTNEGEGSWRSLPKKAGLLRCGKSCRLRWINY	: 60
LjMYB12	1:	MGRAPCCEKVGIIKGRWTAEEDELLTKYIQASGEGSWRSLPKNAGLLRCGKSCRLRWINY	: 60
AtMYB11	61:	LRSDIKRGNI TPEEEDVIVKLHSTLGTRWSTIASNLPGRTDNEIKNYWNSHLSRKLHGYF	: 120
AtMYB12	61:	LRSDLKRGNI TPEEEELVVKLHSTLGNRWSLIAGHLPGRTDNEIKNYWNSHLSRKLHNFY	: 120
AtMYB111	61:	LRRDLKRGNI TSDEEEIIVKLHSLGNRWSLIATHLPGRTDNEIKNYWNSHLSRKYAFT	: 120
LjMYB12	61:	LRGDLKRGNISDEEESLIVKLHASFGRWSLIASHMPGRTDNEIKNYWNSHLSRKLIVYTF	: 120
AtMYB11	121:	RKPT-----VAN-----TVENAPPPP--KRRP-GRTSRSAMKPKFILNPKNHKTPN	: 163
AtMYB12	121:	RKPS- ISQDVS AVIMTNASS----APPPQAKRRL-GRTSRSAMKPK-IHRTKTRKT--	: 170
AtMYB111	121:	AVSGDGHNLLVNDVVLKKSCESSGAKNNNKTKKKKKGRTSRSSMKKHKQMTASQCFS-	: 179
LjMYB12	121:	RGST--TKSIV-D-----TPP----KRRRCGRTSRWAMKKNKTYSLINNDLQN	: 161
AtMYB11	164:	SFKANKSDIVLPPTTTIENGEGDKEDALMVLSSSSLSGAEEPGLGPCGYGDDGDCNP---S	: 220
AtMYB12	171:	-----KKTSAPEPNADVAGADK-EALMVES-----GAEAE LGRPCDYDGD-DCNKNLMS	: 219
AtMYB111	180:	QPKELESDFSEGGQN-GNFEGES-----LGPYEWLDGELERLLSSCVWECT-----	: 224
LjMYB12	162:	QNELEKEDPVARCDVKGSDDILCQEAIN-----VDDDVLSFHEFMDT-----C	: 205
AtMYB11	221:	INGDDGALCLNDDIFDSCFLDDSHAVHVSS-----CESNNVKNSEPYGEMS-----	: 267
AtMYB12	220:	INGDNGVLTFFDDIID--LLLDES DPGHLYTNTTCGGDGE LHNIRDSEGARGFS DTWNQG	: 277
AtMYB111	225:	--SEEAVIGVNDKVKCE-SGDNSSCCVNLF-----EEQGETKIGHVGIIT-----	: 267
LjMYB12	206:	LMEPSGDHDV-DEERES-AVMNDGGSEGVRDT-----CSTVDTVVTSRDSQSKS-----	: 252
AtMYB11	268:	-----VGHKNIETMADDFVDWDFVWREG--QTLWDEKEDLDSVLSRLL	: 308
AtMYB12	278:	NLDCLLQSCPSVESFLNYDHQVNDASTDEFIDWDCVWQEGSDNNLWHEKENPDSMVSLL	: 337
AtMYB111	268:	-----EVDHDMTVEREREGRSFLSSNSNENNDKDWVGLCN-SSEVGFV	: 310
LjMYB12	253:	-----ENHKLHNSCSSNSLG----YSSGLDSVMEEMNREDESSVLSREQ	: 291
AtMYB11	309:	DGEEMESEIRQRDSNDFGEPLDIDEENKMAAWLLS	: 343
AtMYB12	338:	DGDD-EATIGNSNCFNDFGEPLDHDDESALVAWLLS	: 371
AtMYB111	311:	DELLDWEFQGNVTCQSDDLWDLSDIGEITL---	: 342
LjMYB12	292:	NENLLTWLWEDEDWEKDFQIVDPEKHNDMLYWFLS	: 326

Figure 1. Alignment of amino acid sequences of *LjMYB12* (Lj1g3v4863050) with Arabidopsis AtMYB11 (AT3G62610), AtMYB12 (AT2G47460), and AtMYB111 (AT5G49330) in R2R3-MYB subgroup 7 by CLUSTALW. Shaded sequences, conserved residues; bold black underline, R2 and R3 MYB DNA-binding domains; bold gray underline, 'GRTxRSxMK' consensus motif of MYB subgroup 7 of Arabidopsis (Kranz et al. 1998).

Agrobacterium tumefaciens (*Rhizobium radiobacter*) strain EHA105 was used for transformation; 20 mg l⁻¹ acetosyringone was added into the co-cultivation medium; and the concentration of geneticin for transformant selection was changed from 5 to 10 mg l⁻¹ in the shoot induction medium. Transgenic plants expressing *LjMYB12* or *intron-GUS* were named 12OX or IG, respectively. Total DNA was isolated from fresh leaves of transgenic plants and analyzed by Southern blotting as described by Yamada et al. (2014). A statistical analysis was performed to evaluate the population variances among all samples. The Student's *t* test or the Welch's *t* test were used to examine whether population means are significantly different from each sample and control. All statistic data was evaluated significantly different from the control when *p* values were <0.05.

The R2R3-MYB subgroup 7 protein in *L. japonicus* was predicted to consist of 326 residues (Figure 1). The protein shared the highest sequence identity (83%) with AtMYB12 in the R2R3-MYB DNA-binding domain and the consensus motif (Figure 1). Therefore, the R2R3-MYB subgroup 7 gene isolated in present study was designated as *LjMYB12* (AB334529) gene. The nucleotide sequence of *LjMYB12* gene was corresponding to that of one gene ID, Lj1g3v4863050, registered on the database of miyakogusa.jp 3.0 (<http://www.kazusa.or.jp/lotus/>). Subgroup 7 MYBs of Arabidopsis act additively owing to their differential spatial activity in developing seedlings: AtMYB12 controls flavonol biosynthesis mainly in roots, and AtMYB111 controls it mainly in cotyledons (Stracke et al. 2007). The expression of *LjMYB12* was much higher in flowers than in leaves, stems, roots, immature seeds, seedling leaves, and seedling roots, its expression did not differ between seedling leaves and roots (Figure 2). Thus, the spatial expression of *LjMYB12* differed greatly from that of subgroup 7 MYB in Arabidopsis.

As the expression of MYB genes activated by stressors

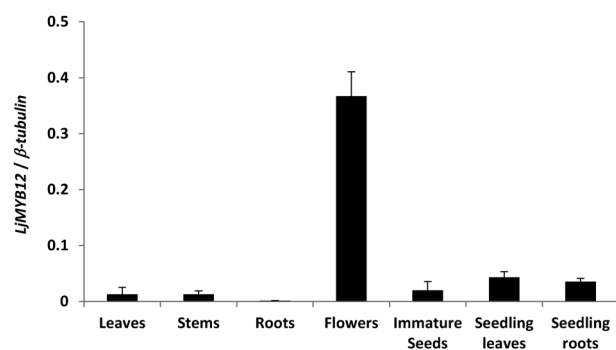


Figure 2. Expression profile of endogenous *LjMYB12* in Gifu B-129. Leaves, stems, roots, and flowers were collected from 14-week-old plants grown at 26°C under a 16/8-h light/dark regime in a growth chamber. Immature seeds were collected from 18-week-old plants. Seedling leaves and roots were collected at 12 days after sowing. Expression levels were determined by normalizing the PCR threshold cycle number of *LjMYB12* to that of β -tubulin. Data are means \pm SD of three biological replicates.

or inducers such as drought, abscisic acid, sucrose, and ultraviolet light (Abe et al. 1997; Stracke et al. 2010; Teng et al. 2005), we examined the expression profile of *LjMYB12* in leaves and roots of seedlings exposed to strong light, methyl jasmonate, sucrose, or nitrogen deficiency. Strong light downregulated *LjMYB12* in seedling leaves (Figure 3A). This is a novel finding of a stress response of R2R3-MYB subgroup 7 in higher plants. Methyl jasmonate did not change the expression of *LjMYB12* (Figure 3B). Sucrose supply and nitrogen deficiency induced *LjMYB12* (Figure 3C and D). Similarly, in Arabidopsis, sucrose supply and nitrogen deficiency weakly induced *AtMYB12* in seedlings (Kranz et al. 1998). *AtMYB12* controls the expression of flavonoid biosynthesis genes such as *CHS*, *CHI*, *F3H*, and *FLS* and the activity of UDP-dependent glycosyltransferase in Arabidopsis (Mehrtens et al. 2005; Stracke et al. 2010). To characterize the spatial and temporal expression of flavonoid biosynthesis genes, we evaluated the expression of 14 genes (*C4H*, *4CL*, *CHS1*, four *CHSs* [*CHS2*, *CHS3*, *CHS-L1*, and *CHS-L2*], *CHI1*, *CHI2*, *CHI3*, *F3H*, *F3'H*, *FLS*, *IFS1*, *IFS2*, *IFR1*, and *DFR2*) in leaves, stems, roots, flowers, immature seeds, seedling leaves, and seedling roots of *L. japonicus*. Flowers, which showed the highest expression of

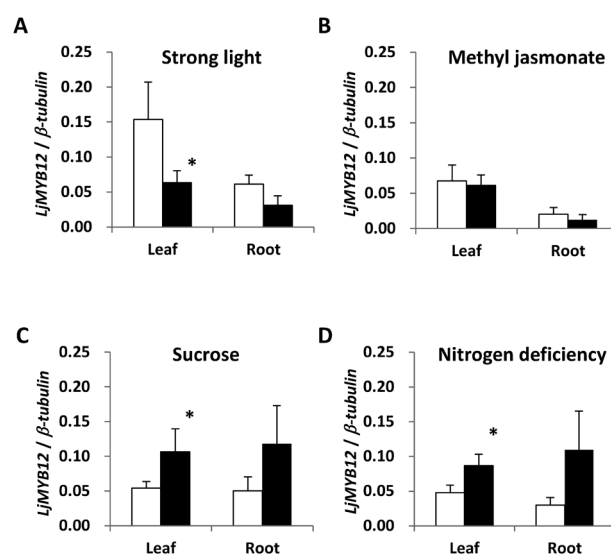


Figure 3. Expression profiles of *LjMYB12* in leaves and roots of Gifu B-129 seedlings exposed to (A) strong light, (B) methyl jasmonate, (C) sucrose, or (D) nitrogen deficiency. □ Control plants: seeds were germinated on basal MS medium containing MS vitamins and 0.3% Gelrite for 4 days in the dark at 26°C, and then grown under continuous light (40–60 μ mol s⁻¹ m⁻²) at 26°C. ■ Treated plants: (A) Seedlings were grown at 26°C under continuous light (250–300 μ mol s⁻¹ m⁻²) for 24 h. (B) Seedlings were treated with 500 μ M methyl jasmonate and 0.1% Triton X-100 for 24 h. (C) Seedlings were grown on basal MS medium containing 100 mM sucrose for 7 days. (D) Seedlings were grown on basal MS medium without nitrogen for 7 days. All expression levels were determined by normalizing the PCR threshold cycle number of *LjMYB12* to that of β -tubulin. Data are means \pm SD of three experimental replicates. *Significantly different at *p* < 0.05.

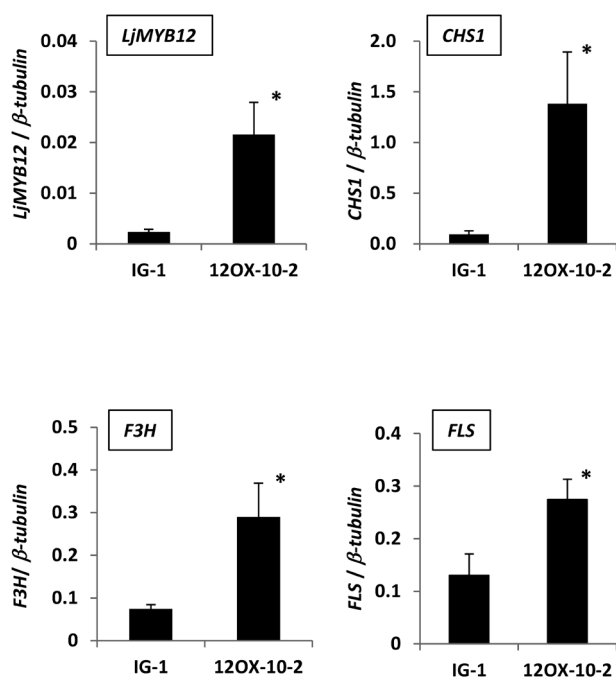


Figure 4. Expression profiles of *LjMYB12*, *CHS1*, *F3H*, and *FLS* in leaves of IG-1 and 12OX-10-2 T_2 plants 7 weeks after sowing. Expression levels were determined by normalizing the PCR threshold cycle number of each gene to that of β -tubulin. Data are means \pm SD of three biological replicates. * Significantly different at $p < 0.05$.

LjMYB12, showed high expression of *C4H*, *4CL*, *CHS1*, *CHSs*, *CHI2*, *F3H*, *F3'H*, *FLS*, and *DFR2* (Supplemental Figure 2). To verify the spatial co-expression of these flavonoid biosynthesis genes and *LjMYB12*, the expression level was evaluated in transgenic *L. japonicus* plants overexpressing *LjMYB12*. Inoculation of explants with *A. tumefaciens* EHA105 harboring pMDC100-*LjMYB12* produced 30 T_0 plants, of which 5 showed $>9\times$ the expression of *LjMYB12* of a control plant which expressed *intron-GUS* (Supplemental Figure 3). However, these T_0 plants did not produce T_1 seeds. On the other hand, four T_0 plants (12OX-2-1, 12OX-6-3, 12OX-10-2, and 12OX-13-1), which showed moderate expression of *LjMYB12*, set T_1 seeds. Although the remaining 21 plants had seed fertility, the expression level of *LjMYB12* was low. High performance liquid chromatography (HPLC) analysis revealed that mature seeds of 12OX10-2 accumulated much more putative flavonoid compounds than control plants (Supplemental Figure 4), indicating that an overexpressing *LjMYB12* gene enhances the biosynthesis of flavonoids. These results suggest that the excessive accumulation of flavonoids caused by very high expression of *LjMYB12* might reduce seed fertility.

For further analyses we used T_2 plants derived from 12OX-10-2, which showed the highest expression level of *LjMYB12* among T_0 plants with seed fertility and normal morphology (Supplemental Figure 5). At least four bands hybridized with the *LjMYB12*-specific probe in Southern blot analysis of the T_2 plants, but only one

band was detected in Gifu B-129 (Supplemental Figure 6). These results indicate that the T_2 plants had multiple copies of *LjMYB12*. We evaluated the expression of *LjMYB12* and flavonoid biosynthesis genes (*CHS1*, *CHSs*, *CHI1*, *CHI2*, *F3H*, *F3'H*, *FLS*, and *DFR2*) in leaves of 7-week-old T_2 plants. The expression of *LjMYB12*, *CHS1*, *F3H*, and *FLS* was significantly higher in 12OX-10-2 T_2 plants than in the control plants (Figure 4). On the other hand, the expression of the other genes was unchanged (Supplemental Figure 7). These results suggest that *CHS1*, *F3H*, and *FLS* are activated by *LjMYB12* in *L. japonicus*.

MYB12 ortholog activity differed between *L. japonicus* and *Arabidopsis*. AtMYB12 activated the transcription of *CHI* in addition to *CHS*, *F3H*, and *FLS* in *Arabidopsis* (Mehrtens et al. 2005), but overexpressed *LjMYB12* did not increase the expression of *CHI* paralogs in transgenic *L. japonicus* plants. Interestingly, *LjMYB12* strongly activated *CHS1* in *L. japonicus*, but did not activate other *CHSs* (Figure 4 and Supplemental Figure 7). Shimada et al. (2007) classified *CHS1* into a different clade from the other *CHS* orthologs in *L. japonicus*. Since the expression of flavonoid biosynthesis genes is also regulated by other R2R3-MYB TFs in addition to the subgroup 7 (Borevitz et al. 2000; Kranz et al. 1998; Nesi et al. 2001; Stracke et al. 2001), molecular characterization of MYB TFs in legumes will reveal how gene duplication affects the regulation of flavonoid biosynthesis.

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