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 (CHI–3), 5 chalcone synthases (CHS1, 2, 3–L1, and 3–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 Ex Taq 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:

![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).](image-url)

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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 genetin 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 R2R3-MYB subgroup 7 protein in Arabidopsis was predicted to consist of 326 residues (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, Ljg3v4863050, registered on the database of miyakogusa.jp 3.0 (http://www.kazusa.or.jp/lotus/). Subgroup 7 MYBs of Arabidopsis act additively owing to one gene ID, Ljg3v4863050, registered on the database of miyakogusa.jp 3.0 (http://www.kazusa.or.jp/lotus/).

As the expression of MYB genes activated by stressors 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 2](image-url)

**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±SD of three biological replicates.

![Figure 3](image-url)

**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: seed 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 μmol m\(^{-2}\) s\(^{-1}\)) at 26°C. ■ Treated plants: (A) Seedlings were grown at 26°C under continuous light (250–300 μmol m\(^{-2}\) s\(^{-1}\)) for 24 h. (B) Seedlings were treated with 500 μM 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±SD of three experimental replicates. * Significantly different at p<0.05.
Characterization of \textit{LjMYB12} gene in \textit{Lotus japonicus}

48

\[ \text{LjMYB12} \]

\[ \text{CHS1} \]

\[ \text{F3H} \]

\[ \text{FLS} \]

Figure 4. Expression profiles of \textit{LjMYB12}, \textit{CHS1}, \textit{F3H}, and \textit{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 \( \beta \)-tubulin. Data are means \( \pm \) SD of three biological replicates. * Significantly different at \( p < 0.05 \).

\textit{LjMYB12} showed high expression of \textit{C4H}, \textit{4CL}, \textit{CHS1}, \textit{CHSs}, \textit{CHI2}, \textit{F3H}, \textit{F3′H}, \textit{FLS}, and \textit{DFR2} (Supplemental Figure 2). To verify the spatial co-expression of these flavonoid biosynthesis genes and \textit{LjMYB12}, the expression level was evaluated in transgenic \textit{L. japonicus} plants overexpressing \textit{LjMYB12}. Inoculation of explants with \textit{A. tumefaciens} EHA105 harboring pMDC100-LjMYB12 produced 30 \( T_0 \) plants, of which 5 showed \( >9 \times \) the expression of \textit{LjMYB12} of a control plant which expressed \textit{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 \textit{LjMYB12}, set \( T_1 \) seeds. Although the remaining 21 plants had seed fertility, the expression level of \textit{LjMYB12} was low. High performance liquid chromatography (HPLC) analysis revealed that mature seeds of 12OX10-2 was low. High performance liquid chromatography (HPLC) analysis revealed that mature seeds of 12OX10-2, which showed the highest expression level of \textit{LjMYB12} among \( T_2 \) plants with seed fertility and normal morphology (Supplemental Figure 5). At least four bands hybridized with the \textit{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 \textit{LjMYB12}. We evaluated the expression of \textit{LjMYB12} and flavonoid biosynthesis genes (\textit{CHS1}, \textit{CHSs}, \textit{CHI1}, \textit{CHI2}, \textit{F3H}, \textit{F3′H}, \textit{FLS}, and \textit{DFR2}) in leaves of 7-week-old \( T_2 \) plants. The expression of \textit{LjMYB12}, \textit{CHS1}, \textit{F3H}, and \textit{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 \textit{CHS1}, \textit{F3H}, and \textit{FLS} are activated by \textit{LjMYB12} in \textit{L. japonicus}.

\textit{MYB12} ortholog activity differed between \textit{L. japonicus} and Arabidopsis. \textit{AtMYB12} activated the transcription of \textit{CHI} in addition to \textit{CHS}, \textit{F3H}, and \textit{FLS} in Arabidopsis (Mehretts et al. 2005), but overexpressed \textit{LjMYB12} did not increase the expression of \textit{CHI} paralogs in transgenic \textit{L. japonicus} plants. Interestingly, \textit{LjMYB12} strongly activated \textit{CHS1} in \textit{L. japonicus}, but did not activate other \textit{CHS}s (Figure 4 and Supplemental Figure 7). Shimada et al. (2007) classified \textit{CHS1} into a different clade from the other \textit{CHS} orthologs in \textit{L. japonicus}. Since the expression of flavonoid biosynthesis genes is also regulated by other \( R2R3-MYB \) TFs in legumes will reveal how gene duplication affects the regulation of flavonoid biosynthesis.

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References


