Running Title: MYB regulating anthocyanin biosyntheses in *Lilium*

Corresponding Author:

Masumi Yamagishi, PhD

Research Faculty of Agriculture, Hokkaido University, Kita-ku, Sapporo 060-8589, Japan

Tel/Fax: +81-11-706-3846.

e-mail: yamagisi@res.agr.hokudai.ac.jp

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Two R2R3-MYB Genes, Homologues of Petunia AN2, Regulate Anthocyanin Biosyntheses in Flower Tepals, Tepal Spots and Leaves of Asiatic Hybrid Lily

Masumi Yamagishi[1,*], Yoshihiro Shimoyamada[2], Takashi Nakatsuka[3] and Kiyoshi Masuda[1]

[1] Research Faculty of Agriculture, Hokkaido University, Kita-ku, Sapporo 060-8589, Japan

[2] Graduate School of Agriculture, Hokkaido University, Kita-ku, Sapporo 060-8589, Japan

[3] Iwate Biotechnology Research Center, Narita, Kitakami, Iwate 024-0003, Japan

Abbreviations: bHLH, basic helix-loop-helix; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcription-PCR

* Corresponding author: E-mail, yamagisi@res.agr.hokudai.ac.jp

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(Abstract)

Anthocyanins are secondary metabolites that contribute to colors of flowers, fruits and leaves. Asiatic hybrid lily (*Lilium* spp.) accumulates cyanidin anthocyanins in flower tepals, tepal spots and leaves of juvenile shoots. To clarify their regulation mechanisms of anthocyanin pigmentation, two full-length cDNA of *R2R3-MYB* (*LhMYB6* and *LhMYB12*) were isolated from the anthocyanin-accumulating tepals of cultivar 'Montreux'. Analysis of the deduced amino acid sequences indicated they have a homology with petunia AN2, of which homologous sequences had not been isolated in species of monocots. Yeast two-hybrid analysis showed that LhMYB6 and LhMYB12 interacted with the *Lilium* hybrid basic helix-loop-helix 2 (LhbHLH2) protein. Transient expression analysis indicated that co-expression of *LhMYB6* and *LhbHLH2* or *LhMYB12* and *LhbHLH2*, introduced by a microprojectile, activated the transcription of anthocyanin biosynthesis genes in lily bulbscales. Spatial and temporal transcription of *LhMYB6* and *LhMYB12* was analyzed. The expression of *LhMYB12* corresponded well with anthocyanin pigmentation in tepals, filaments and styles, and that of *LhMYB6* correlated with anthocyanin spots in tepals and light-induced pigmentation in leaves. These results indicate that *LhMYB6* and *LhMYB12* positively regulate anthocyanin biosynthesis and determine organ- and tissue-specific accumulation of anthocyanin.

Keywords: Anthocyanin pigmentation - basic helix-loop-helix (bHLH) - Cyanidin 3-*O*-β-rutinoside - *Lilium* spp. - Tepal spots - Transcription factor

Introduction

Genus *Lilium* comprises more than 90 species (Asano 1989) and is classified into sections (Comber 1949, Smyth et al. 1989). Most species of section Sinomartagon, such as *L. dauricum, L. maculatum, L. concolor, L. leichtlinii, L. davidii* and *L. cernuum,* are distributed in East Asia. The Asiatic hybrid lily, one of the most popular ornamental plants world-wide, is derived from inter-specific crosses of species of section Sinomartagon (Leslie 1982). Asiatic hybrid lily cultivars have high variation in tepal colors: yellow, orange, red, pink and white. Carotenoids accumulate in yellow and orange tepals; yellow carotenoids such as antheraxanthin, violaxanthin and lutein are in yellow tepals (Yamagishi et al. in press), and capsanthin is the major pigment in orange tepals (Deli et al. 1998). Pigments accumulated in pink tepals are anthocyanins (Nørbæk and Kondo 1999, Abe et al. 2002). Accumulation of both anthocyanins and capsanthin pigments the tepals red (Yamagishi et al. in press). Asiatic hybrid lily cultivars often have dark red spots on their interior surfaces of tepals (Fig. 1). Pigments accumulated in tepal spots are anthocyanins (Abe et al. 2002).

Anthocyanins are found widely in plant species and are responsible for the purple, blue and pink coloration of plant parts. Anthocyanins provide color to flowers and fruits needed to attract pollinators and seed-dispersing animals (Winkel-Shirley 2001, Schaefer et al. 2004, Grotewold 2006). Anthocyanins help to protect plants from various stresses, such as strong sunlight and active oxygen species (Nagata et al. 2003, Gould 2004). Three basic groups of anthocyanin pigments are in higher plants: derivatives of pelargonidin, of cyanidin and of delphinidin (Schwinn and Davies 2004). *Lilium* species contain cyanidin 3-*O*-β-rutinoside as a major anthocyanin and cyanidin 3-*O*-β-rutinoside-7-*O*-β-glucoside as a minor anthocyanin (Nørbæk and Kondo 1999).

Asiatic hybrid lily cultivar ‘Montreux’, which was mainly used in this study, contains only cyanidin 3-*O*-β-rutinoside in tepals and tepal spots (Abe et al. 2002).

Inheritance of tepal anthocyanin and anthocyanin spots was analyzed in Asiatic hybrid lily using F_1 plants derived from a cross between cultivars ‘Montreux’ (pink tepals with spots) and ‘Connecticut King’ (yellow [no anthocyanin] tepals without spots) (Abe et al. 2002). F_1 plants with or without tepal anthocyanin segregated in a ratio of 1 to 1, indicating that a single major gene controls this trait, and the number of anthocyanin spots showed continuous distribution in the F_1 population, indicating that this trait is a quantitative trait. After trait loci mapping, the loci of tepal anthocyanin and of anthocyanin spots were mapped on the different linkage groups of Asiatic hybrid lily (Abe et al. 2002). These results indicate that these pigmentations are controlled independently even though tepal anthocyanin and anthocyanin spots consist of the same pigment. Because yellow tepal cultivar ‘Connecticut King’ has the ability to biosynthesize anthocyanin in organs other than tepals, such as in anthers and light-exposed bulbscales, and can express anthocyanin biosynthesis genes *chalcone synthase* (*CHS*) and *dihydroflavonol 4-reductase* (*DFR*), transcription factors may mainly regulate tepal anthocyanin and anthocyanin spots in Asiatic hybrid lily (Nakatsuka et al. 2003).

The biochemistry and enzymology of the anthocyanin biosynthesis pathway is well understood (Winkel-Shirley 2001, Schwinn and Davies 2004). The activity of anthocyanin biosynthesis enzymes is mainly controlled at the transcriptional level and is regulated by interactions between R2R3-MYB and basic-helix-loop-helix (bHLH) transcription factors (Mol et al. 1998, Schwinn and Davies 2004, Koes et al. 2005), such as between ZmC1 (R2R3-MYB) and ZmR (bHLH) in maize kernels (Dooner et al. 1991), GhMYB10 (R2R3-MYB) and GhMYC1 (bHLH) in *Gerbera hybrida* flowers

(Elomaa et al. 1998, 2003), and PhAN2 (R2R3-MYB) and PhAN1 (bHLH) in petunia flowers (Spelt et al. 2000). To determine whether R2R3-MYB and bHLH proteins regulate anthocyanin biosynthesis in Asiatic hybrid lily, we isolated and characterized *bHLH* genes in a previous study (Nakatsuka et al. 2009) and *R2R3-MYB* genes in this study.

In Asiatic hybrid lily, two *bHLH* genes *LhbHLH1* and *LhbHLH2* are expressed in some organs, including tepals; *LhbHLH1* is a *PhJAF13* homologue and *LhbHLH2* is a *PhAN1* homologue. Petunia JAF13 is a second bHLH protein that interacts with PhAN2, but is unlikely to participate directly in regulating the transcription of anthocyanin biosynthesis genes because *PhJAF13* does not complement petunia *an1* mutants (Quattrocchio et al. 1998, Spelt et al. 2000). In Asiatic hybrid lily, *LhbHLH2* is predominantly correlated to anthocyanin biosynthesis because of the transcriptional profile in organs accumulating anthocyanin (Nakatsuka et al. 2009). However, LhbHLH2 is not responsible for the tepal color difference among the cultivars because the transcription pattern of *LhbHLH2* in ‘Montreux’ and ‘Connecticut King’ tepals is nearly the same (Nakatsuka et al. 2009).

R2R3-MYB transcription factors that regulate anthocyanin pigmentation are often divided into two subgroups due to their sequence homology: one subgroup includes *PhAN2* in petunia and *PAP1* in *Arabidopsis* (hereinafter referred to as the *AN2* subgroup), and another subgroup includes *ZmC1* and *ZmPl* in maize (hereinafter referred to as the *C1* subgroup) (Stracke et al. 2001, Allan et al. 2008). The *AN2* and *C1* subgroups are respectively categorized as subgroups 6 and 5 in the *Arabidopsis R2R3-MYB* gene family (Kranz et al. 1998, Stracke et al. 2001) and as subgroups N09 and N08 in the *R2R3-MYB* gene family of *Arabidopsis* and rice (Jiang et al. 2004). Most *R2R3-MYB* genes that regulate anthocyanin biosynthesis in flowers and fruits of

eudicots species are in the *AN2* subgroup, and *R2R3-MYB* genes in monocots that regulate anthocyanin pigmentation in leaves and kernels of Poaceae (Gramineae) species and flowers in orchid *Oncidium* (*OgMYB1*, Chiou and Yeh 2008), all belong to the *C1* subgroup (Allan et al. 2008). To the best of our knowledge, the *An2* subgroup *R2R3-MYB* genes from kernels and flowers of monocots species have not been isolated previously.

In flower and ornamental plants, flower color is an important consideration in consumer choice. Much interest has been in cultivars bearing flowers with altered color, hues and patterns. Thus, understanding the genetic basis that changes colors, hues and patterns should aid breeding new cultivars. In this study, two cDNA clones encoding *R2R3-MYB* transcriptional factors were isolated from pink tepals of Asiatic hybrid lily, and their biochemical properties and spatial and temporal expressions were investigated to know the regulatory system of anthocyanin biosynthesis and to determine which transcription factors are responsible for color differences among the cultivars.

Results

Cloning R2R3-MYB transcription factors from lily tepals

A PCR-based method was used to isolate *R2R3-MYB* transcription factor genes from tepals of Asiatic hybrid lily cultivar ‘Montreux’. We originally designed degenerated primers (dMYBf and dMYBr) based on the conserved amino acid sequences in *C1* and *AN2* subgroup *R2R3-MYB* genes, and amplified eight groups of 128 bp sequences showing homology with *R2R3-MYB* (data not shown). Because two of the eight showed homology with the *PhAN2* sequence, full-length cDNA sequences of the two were determined by rapid amplification of cDNA ends (RACE)-PCR. Sequence homologous

to *ZmC1* gene was not included in the eight groups.

The two full-length cDNA sequences, *LhMYB6* and *LhMYB12*, encoded 276 and 246 amino acids, respectively. An amino acid alignment of 26 R2R3-MYB sequences in lily and other species (Supplementary Fig. S1) showed that the region of R2R3 repeats was highly conserved, but downstream of this region was divergent both in sequence and length. All AN2 and C1 subgroup sequences and lily sequences contained the motif [D/E]Lx$_2$[K/R]x$_3$Lx$_6$Lx$_3$R in the R3 repeat necessary for interactions with R-like bHLH proteins (Zimmermann et al. 2004, Takos et al. 2006). In the variable region, the small motif [K/R]P[Q/R]P[Q/R] was conserved in AN2 subgroup R2R3-MYB and in LhMYB6 and LhMYB12 sequences. This motif was part of the motif previously reported as KPRPR[S/F]F (motif 6, Stracke et al. 2001) and [K/R]Px$_3$[K/T][F/Y] (Takos et al. 2006). The short conserved sequence KAx[K/R]C[S/T] was recognized in the C1 subgroup, but this sequence did not appear in LhMYB6 and LhMYB12 sequences. Incomplete direct repeats were in the variable region of the LhMYB6 sequence, and so LhMYB6 had two [K/R]P[Q/R]P[Q/R] motifs (Supplementary Fig. S1). A phylogenetic tree, drawn by using full-length amino acid sequences (Fig. 2), showed that LhMYB6 and LhMYB12 formed a cluster with sequences of the AN2 subgroup. Other monocots sequences of R2R3-MYB regulating anthocyanin pigmentation, such as ZmPl and ZmC1 in maize and OgMYB1 in orchid, were all included in the cluster of C1 subgroup. When only amino acid sequences at R2R3 repeats were used to construct a phylogenetic tree, a similar result was obtained, that is, the AN2 subgroup sequences and the lily sequences formed one cluster, and the Cl subgroup sequences formed another cluster (data not shown).

Protein-protein interaction of LhMYB6 and LhMYB12 with LhbHLH2

Transcription of anthocyanin biosynthesis genes is mainly regulated by interactions between R2R3-MYB and bHLH transcription factors (Schwinn and Davies 2004, Koes et al. 2005). Both LhMYB6 and LhMYB12 contained a motif necessary for interactions with R-like bHLH proteins (Supplementary Fig. S1). Thus, whether LhbHLH2 protein (Nakatsuka et al. 2009) interacts with LhMYB6 or LhMYB12 protein was investigated using a GAL4-based yeast two-hybrid system. The GAL4 DNA-binding domain-fused LhbHLH2 and activation domain-fused LhMYB6 or LhMYB12 were introduced into yeast (Fig. 3). Only yeast harboring the combination of LhMYB6 and LhbHLH2 and of LhMYB12 and LhbHLH2 survived on a quadruple dropout medium. When 3-amino-1, 2, 4-triazole (3-AT), which is a competitive inhibitor of His3 protein, was supplemented to the medium, the growth of yeast harboring LhMYB12 and LhbHLH2 was lower than that of yeast harboring LhMYB6 and LhbHLH2. Therefore, LhMYB6 and LhbHLH2, and LhMYB12 and LhbHLH2, would form a heterodimer by protein-protein interaction, and the interaction between the former two proteins is stronger than that between the latter two proteins.

Ability of LhMYB6 and LhMYB12 to activate transcription of anthocyanin biosynthesis genes determined by transient expression assay

Because LhMYB6 and LhMYB12 showed homology with the AN2 subgroup R2R3-MYB proteins, whether expressional activation of *LhMYB6* and *LhMYB12* could stimulate transcription of anthocyanin biosynthesis genes was investigated by a transient expression assay. Constructs of 35S-driven *LhMYB6* or *LhMYB12* were bombarded into white bulbscales of ‘Montreux’ together with or without a 35S-driven *LhbHLH2* construct. At 48 h after the bombardment, transcription of *LhDFR*, *LhCHSa* and *LhCHSb* was detected by RT-PCR in the bombarded organ where the expression of

LhbHLH2 and *LhMYB6*, and *LhbHLH2* and *LhMYB12*, appeared together (Fig. 4). The bombardment of a 35S-driven *LhbHLH2* construct only did not activate transcription of *LhDFR*, *LhCHSa* and *LhCHSb* (data not shown). These results indicate that LhMYB6 and LhMYB12 activate the transcription of anthocyanin biosynthesis genes and that LhbHLH2 protein is necessary when LhMYB6 and LhMYB12 activate the transcription.

Temporal and spatial transcription of *LhMYB6* and *LhMYB12*

To clarify which transcripts of *LhMYB6* or *LhMYB12* accumulated in anthocyanin pigmented-organs, their temporal and spatial transcription was evaluated by quantitative RT-PCR. The temporal transcription of *LhMYB6* and *LhMYB12* during tepal development of 'Montreux' (basal and upper parts of tepals) and 'Connecticut King' (basal part of tepals) was examined. Flowers were divided into five developmental stages based on mainly tepal pigmentation (Fig. 1): stage 1 (about 3 cm in bud length), no pigmentation; stage 2 (about 4 cm), spots visible on the basal part; stage 3 (about 6 cm), tepal pigmentation began; stage 4 (one day before anthesis, about 7 cm), the content of tepal anthocyanin was highest; stage 5 (about 8 cm), flowers opened. Nakatsuka et al. (2003) shows temporal changes of anthocyanin contents in tepals. Midribs of tepals showed anthocyanin pigmentation on the abaxial side at all stages, and so midribs were not included in this study when gene expression was analyzed. No anthocyanin pigment was in 'Connecticut King' tepals (data not shown), of which the developmental stages were judged based on tepal length.

In *LhMYB6*, the transcriptional profile of 'Montreux' tepals differed between the basal and the upper parts. The transcription was higher at stages 1, 2 and 5 than at stages 3 and 4 in the basal part, and the transcriptional change was low during all tepal stages

in the upper part (Fig. 5A). In ‘Connecticut King’ tepals, transcription of LhMYB6 was low and did not change during tepal development (Fig. 5A).

The transcript of *LhMYB12* accumulated at tepal stages 3, 4 and 5 and peaked at stage 4 in ‘Montreux’ (Fig. 5B). This transcription pattern correlated well with anthocyanin accumulation in tepals, which began at stage 3 and peaked at stage 4 (Fig. 1) (Nakatsuka et al. 2003). The transcriptional level was higher in the basal part than in the upper part. Nakatsuka et al. (2003) shows that anthocyanin concentration is also higher in the basal part than in the upper part. No transcription of *LhMYB12* was detected in all tepal stages of ‘Connecticut King’. These results indicate that LhMYB12 regulates biosynthesis of tepal anthocyanin.

The transcription of *LhDFR* was investigated (Fig. 5C). In ‘Montreux’, a high level of transcription was detected in tepal stages 3, 4 and 5 in the basal and the upper parts. Tepal stage 1 showed very low amounts and stage 2 showed low amounts of transcripts in the basal part, but no transcription was detected in stages 1 and 2 in the upper part. Transcripts of *LhDFR* were not detected in ‘Connecticut King’ at any developmental stage.

At stage 2, a spot pigment was visible in ‘Montreux’, and transcription of *LhDFR* was detected only in the basal part where spots were present, that is, the *LhDFR* expression at stage 2 correlated with anthocyanin biosynthesis in the spots. *LhMYB12* transcription was not detected at stage 2, indicating that LhMYB12 does not contribute to *LhDFR* expression at this stage and to spot pigmentation. Instead, *LhMYB6* transcription was high at stages 1 and 2 only in the basal part, suggesting that LhMYB6 stimulates *LhDFR* expression and regulates spot pigmentation.

Quantitative RT-PCR was done to clarify the spatial expression pattern of *LhMYB6* and *LhMYB12* genes in various organs of ‘Montreux’. Flower organs were collected

from the stage 4 flowers. Anthocyanin pigment was in the anthers, filaments, styles and tepals. *LhMYB6* transcription was detected in all organs and was relatively high in ovaries (Fig. 5D) that showed no anthocyanin pigmentation. The *LhMYB12* transcript accumulated in tepals, filaments and styles (Fig. 5E). Transcription of *LhMYB12* was not detected in anthers, but anthocyanin pigment accumulated in them. *LhDFR* transcription was detected in tepals, filaments and styles, but not in anthers, ovaries and leaves (Fig. 5F).

Transcription of *LhMYB6* and *LhMYB12* in F1 plants

The temporal transcriptional profile in tepals suggests that LhMYB6 regulates spot pigmentation (Fig. 5A). To confirm this role of LhMYB6, transcription of *LhMYB6* was analyzed using F_1 plants derived from the cross between 'Montreux' (pink tepals with spots) and 'Connecticut King' (yellow tepals without spots). Because tepal anthocyanin and tepal spots are inherited independently (Abe et al. 2002), pink tepal individuals without tepal spots and yellow tepal individuals with spots segregated in F_1 plants. Stage 2 tepals were used for this analysis. Real time RT-PCR showed *LhMYB6* transcription in F_1 plants with spots was higher than in F_1 plants without spots ($t = 7.87$, $P < 1\%$) (Fig. 6), indicating that *LhMYB6* regulates spot pigmentation. *LhMYB12* transcription was not detected in these F_1 plants at this stage (data not shown), indicating that LhMYB12 does not contribute to spot pigmentation.

To confirm the role of LhMYB12 regulating tepal pigmentation, its transcription in tepals of F_1 plants was analyzed at tepal stage 5. F_1 plants with yellow or white tepals (no anthocyanin) showed no transcription, but F_1 plants with pink-tepals (by anthocyanin) showed *LhMYB12* transcription (Fig. 7), indicating that *LhMYB12* regulates tepal pigmentation.

Effect of light on *LhMYB6* transcription in leaves

Because juvenile lily shoots often accumulate anthocyanin at sprouting, we previously analyzed the effect of light on anthocyanin accumulation and transcription of *LhbHLH1* and *LhbHLH2* in juvenile leaves (Nakatsuka et al. 2009). When Asiatic hybrid lily cultivar 'Vivaldi' was grown in the dark, the anthocyanin content was very low in leaves, but the anthocyanin content rapidly increased during 2 d of light exposure and peaked at 4 d of light exposure. At 6 and 8 d of light treatment, the amount of anthocyanin pigment declined and was nearly the same level as that in the dark (Nakatsuka et al. (2009) shows temporal changes of anthocyanin content in leaves). In this study, transcriptions of *LhMYB6*, *LhMYB12* and *LhDFR* were analyzed using the same leaf materials as in the previous study (Fig. 8). The expression of *LhMYB6* was not detected in the dark (0 d), but it increased rapidly during 2 d of light exposure. Then the transcription declined rapidly and was low at 6 and 8 d of light treatment. A similar transcription profile was detected for *LhDFR*, that is, its transcription was not detected in the dark (0 d), increased rapidly during 2 d of light exposure and then declined rapidly. In contrast to the *LhMYB6* expression, transcription of *LhDFR* was nearly zero at 8 d of light treatment. Transcription of *LhMYB12* was not detected during the 8 d (data not shown). These results indicate that the expression of *LhMYB6*, but not of *LhMYB12*, correlates well with light-induced anthocyanin accumulation in sprouting lily shoots.

Discussion

Flower pigmentation in Asiatic hybrid lily is temporally and spatially controlled by the

expression of anthocyanin biosynthesis genes (Nakatsuka et al. 2003). Because transcription of anthocyanin biosynthesis genes is mainly regulated by interaction between R2R3-MYB and bHLH transcription factors (Schwinn and Davies 2004, Koes et al. 2005), we characterized *LhbHLH1* (*Jaf13* homologue) and *LhbHLH2* (*AN1* homologue) genes previously (Nakatsuka et al. 2009) and *R2R3-MYB* genes in this study. Partial cDNA sequences of *R2R3-MYB* were isolated using degenerate primers designed from conserved regions of R2R3 repeats. Such a PCR-based strategy was successfully applied to maize (Rabinowicz et al. 1999), *Arabidopsis* (Romero et al. 1998), orchid (Wu et al. 2003) and gentian (Nakatsuka et al. 2008). After RACE-PCR, full-length cDNA sequences of *R2R3-MYB* genes, *LhMYB6* and *LhMYB12*, were isolated. Phylogenetic analysis (Fig. 2) and the presence of a conserved motif in the variable region (Supplementary Fig. S1) indicated they belong to the *AN2* subgroup. This is the first report of the *AN2* subgroup *MYB* genes isolated from monocots species. However, we do not know whether the *AN2* subgroup *MYB* sequences are rare or common in monocots because *R2R3-MYB* genes controlling flavonoid biosynthesis have been isolated in only three families in monocots; family Liliaceae (the *AN2* subgroup, this study), Orchidaceae (*OgMYB1* in orchid, the *C1* subgroup, Chiou and Yeh 2008) and Poaceae (*ZmC1*, *ZmPl*, *OsC1*, etc., the *C1* subgroup, Cone et al. 1993, Reddy et al. 1998). Isolation of such *R2R3-MYB* genes from other families of monocots is necessary to answer this question. The rice genome has no *R2R3-MYB* sequence homologous with *AN2* (Jiang et al. 2004, Matus et al. 2008), suggesting that during evolution the rice genome lost *AN2* homologous genes after separation of rice-lily lineages.

The series of evidence indicates that *LhMYB6* and *LhMYB12* are transcription factors regulating anthocyanin biosynthesis in Asiatic hybrid lilies. (1) In a transient

assay, *LhMYB6* and *LhMYB12* genes together with the *LhbHLH2* gene activated transcription of anthocyanin biosynthesis genes in lily tissues. (2) LhMYB6 and LhMYB12 interacted with LhbHLH2 protein in a yeast two-hybrid system, like R2R3-MYB proteins that regulate anthocyanin biosynthesis in other species interact with bHLH proteins. (3) *LhMYB6* and *LhMYB12* were expressed in organs where anthocyanin pigment accumulated, with some exceptions such as in anthers (we discuss this exception later).

LhbHLH2 protein was necessary when LhMYB12 activated the transcription of anthocyanin biosynthesis genes (Fig. 4). However, the interaction of LhMYB12 with LhbHLH2 was weaker than that between LhMYB6 and LhbHLH2 in yeast two-hybrid analysis (Fig. 3). Do these results suggest that another bHLH protein that specifically interacts with LhMYB12 is expressed in lily flowers, or does LhMYB12 make up for the weak interaction with LhbHLH2 protein by its high expression, which was about 10 to 100 times higher than that of LhMYB6? Further study is necessary to answer this question.

Although both LhMYB6 and LhMYB12 regulated anthocyanin biosynthesis, their transcriptional profiles differed in flower organs (Fig. 5). *LhMYB12* transcript appeared in tepal stages 3, 4 and 5 in both basal and upper parts of ‘Montreux’ tepals. This increase in *LhMYB12* transcript was accompanied by accumulation of anthocyanin pigment and *LhDFR* expression in tepals. Similarly, *LhMYB12* transcript was detected in filaments and styles where *LhDFR* transcript and anthocyanin accumulated. These results indicate that LhMYB12 regulates anthocyanin pigmentation in tepals, filaments and styles. However, transcription of *LhMYB6* was high at tepal stages 1 and 2 in only the basal part of ‘Montreux’ that had spot pigmentation. Transcription of *LhMYB6* in tepals was higher in F_1 plants with tepal spots than in F_1 plants without tepal spots (Fig.

6). These results indicate that LhMYB6 regulates pigmentation in tepal spots. That is, two *R2R3-MYB* genes regulate the anthocyanin-pigmentation, one gene in tepals, filaments and styles, and the other gene in tepal spots. Although the same anthocyanin cyanidin 3-*O*-β-rutinoside is accumulated in tepals and tepal spots (Abe et al. 2002), its biosynthesis is regulated independently.

LhMYB12 transcripts accumulated in ‘Montreux’ tepals, but their expression was not detected in ‘Connecticut King’ tepals (Fig. 5). *LhMYB12* transcript was detected in pink tepals of F_1 plants but not in yellow or white tepals of F_1 plants (Fig. 7), indicating that *LhMYB12* determines whether tepals will be pigmented by anthocyanin or not. Because the presence or absence of tepal anthocyanin is controlled by the single dominant locus *LILIUM ANTHOCYANIN PIGMENTATION* (*LAP*) (Abe et al. 2002), *LhMYB12* should be on the *LAP* locus. To proof this, mapping *LhMYB12* onto the linkage map of ‘Montreux’ (Abe et al. 2002) is necessary.

LhMYB6 regulated spot pigmentation. When spots developed on tepals of ‘Montreux’, cell division occurred on the inside of tepals to form small mounds on the tepal surfaces followed by pigment accumulation. Interestingly, pigment accumulated in not only epidermis cells but also in cells inside tepals (M. Yamagishi, unpublished results). However, the interior surfaces of ‘Connecticut King’ tepals were smooth with no mounds or projections. Thus, cell division to form spots may not occur in ‘Connecticut King’ tepals. Because a small amount of *LhMYB6* transcripts was detected in ‘Connecticut King’ tepals (Fig. 5A), ‘Connecticut King’ should have the *LhMYB6* gene. However, because cell division to form spots did not occur in this cultivar, *LhMYB6* transcription was not activated in the basal part of tepals. That is, spot pigmentation regulated by LhMYB6 should be downstream of the event of cell division.

Species in such genera as *Tricyrtis* and *Alstroemeria* in monocots and

Rhododendron in eudicots form spots (or blotches) on their interior surfaces of tepals or petals. As no precise examination on spot formation in these species may exist, we do not know whether these spots are anatomically the same. Further study of spot formation in lilies and other plant species is necessary because tepal spots are an important character that varies color pattern in flowers.

Many *Lilium* species and cultivars accumulate anthocyanin in their juvenile shoots, and this pigment usually disappears when leaves expand well. Such anthocyanin is thought to protect plants from strong sunlight until sufficient pigments necessary for photosynthesis accumulate (Gould 2004). Light-induced expression of *LhMYB6* in leaves (Fig. 8) indicates that LhMYB6 is responsible for biosynthesis of such anthocyanin in vegetative organs. *LhMYB12* showed no light-induced expression in leaves. Transcription of *bHLH* genes was also examined (Nakatsuka et al. 2009) using the same materials as those in this study. The *LhbHLH1* transcript slightly accumulated in leaves at 0 d and was highly detected at 2, 4, 6 and 8 d of treatment. The expression of *LhbHLH2* was not detected in the dark (at 0 d), but the *LhbHLH2* transcript rapidly accumulated in response to light at 2 d and then declined to some extent. In contrast to *LhMYB6* whose transcription was low at 6 and 8 d of light treatment (Fig. 8), transcription of *LhbHLH1* and *LhbHLH2* was detected at high levels at 6 and 8 d of light treatment even though the anthocyanin contents were nearly zero at 8 d of treatment (Nakatsuka et al. 2009), indicating that *LhMYB6* limits light-induced anthocyanin pigmentation in leaves more closely than *LhbHLH1* and *LhbHLH2*.

No transcription of *LhMYB6* in leaves was detected in the dark (at 0 d), but a small amount (relative expression of about 0.01-0.02) of *LhMYB6* transcripts was detected at 6 and 8 d of light exposure (Fig. 8). Such small levels of *LhMYB6* transcription also appeared in flower organs, such as anthers, filaments and styles of ‘Montreux’ (Fig. 5D)

and in the upper part of ‘Montreux’ tepals and the basal part of ‘Connecticut King’ tepals (Fig. 5A). These results indicate that the expression of *LhMYB6* is constantly induced by light. However, *LhMYB6* did not activate *LhDFR* expression when its relative transcription was about 0.01-0.02 because *LhDFR* expression was very low at 8 d of light exposure. When light exposure began, the level of *LhMYB6* transcription was high enough to induce *LhDFR* expression. Light-induced expression of *R2R3-MYB* genes has been reported for *MdMYB1* in apple (Takos et al. 2006) and *PAP1* and *PAP2* in *Arabidopsis* (Cominelli et al. 2008). The expression of these *R2R3-MYB* genes is induced by light exposure, but the expression keeps the levels high enough to induce expression of anthocyanin biosynthesis genes under light. That is, the response of *LhMYB6* to light exposure is not completely the same as that of *MdMYB1*, *PAP1* and *PAP2*.

Although anthocyanin accumulated in anthers, *LhMYB12* transcripts were not detected, and transcription of *LhMYB6* was not high in anthers (Fig. 5). The expression pattern of structural genes and other regulatory genes is unique in lily anthers. Among three *CHS* genes, *LhCHSa* and *LhCHSb*, which are major *CHS* genes expressed in tepals, are not expressed in anthers, and only *LhCHSc* is expressed in anthers (Nakatsuka et al. 2003). Transcription of the *LhDFR* gene that was high in tepals, filaments and styles was not detected in anthers (Fig. 5), suggesting that another *DFR* gene acts in anthers. Few transcriptions of both *LhbHLH1* and *LhbHLH2* are detected in anthers (Nakatsuka et al. 2009). Thus, different regulatory genes and structural genes should exist to biosynthesize anthocyanin in lily anthers. The *LhDFR* transcript in anthers was detected by northern blot analysis by Nakatsuka et al. (2003) but it was not detected in this study by RT-PCR, indicating that a putative *DFR* gene that acts in lily anthers has high homology with *LhDFR* but does not have sequences of the primers

used in this study. In other species, regulation of anthocyanin biosynthesis is also complicated in anthers. For example, *PhAN4* controls anthocyanin production in petunia anthers instead of *PhAN2* (Spelt et al. 2000) by regulating the expression of *PhCHSJ*, but not of *PhCHSA* (Quattrocchio et al. 1993).

The expression of *LhMYB6* was relatively high in ovaries (Fig. 5). Accumulation of anthocyanin was not detected in ovaries, but flavonols accumulated in them (M. Yamagishi, unpublished result). Flavonols are believed to be essential for pollen germination and tube growth (Napoli et al. 1999), and flavonols and the transcripts of flavonol biosynthesis genes accumulate in developing ovules and placentas of ovaries in *Antirrhinum majus* (Moyano et al. 1996). However, R2R3-MYB transcription factors different from those regulating anthocyanin biosynthesis usually regulate flavonol accumulation (Moyano et al. 1996, Stracke et al. 2007). Thus, LhMYB6 may not contribute directly to flavonol biosynthesis in lily ovaries. The function of LhMYB6 in ovaries remains to be determined.

In conclusion, this study showed that newly identified AN2 subgroup MYB transcription factors, LhMYB6 and LhMYB12, definitely participate in regulating anthocyanin biosynthesis in Asiatic hybrid lily. LhMYB6 is multifunctional regulating both spot pigmentation in tepals and light-induced pigmentation in leaves. LhMYB12 regulates anthocyanin biosynthesis in flower tepals, filaments and styles. That is, each R2R3-MYB regulates anthocyanin biosyntheses in organs and tissues. In the *AN2* subgroup R2R3-MYB, two or more genes are often included in a single plant species, such as *PAP1*, *PAP2*, *AtMYB113* and *AtMYB114* in *Arabidopsis* (Gonzalez et al. 2008), *AmROSEA1* and *AmROSEA2* in snapdragon (Schwinn et al. 2006), and *VlMYBA1* and *VlMYBA2* in grape (Walker et al. 2007). These sets of genes usually show functional redundancy acting in the same organs for a similar period. One exception is

AmVENOSA in snapdragon, which pigments the epidermal layer in only regions overlying the vascular tissues of petals, whereas AmROSEA1 and AmROSEA2 pigment the epidermal layer in wider regions of petals (Schwinn et al. 2006). Thus, compared with these genes, *LhMYB6* and *LhMYB12* have diverse functions. A more precise examination of *LhMYB6* and *LhMYB12* should be valuable to determine how they gained such diverse functions.

Materials and Methods

Plant materials

Asiatic hybrid lily (*Lilium* spp.) cultivars 'Montreux', 'Connecticut King' and 'Vivaldi' were used. For the analysis of temporal gene expression, tepals in field-grown 'Montreux' and 'Connecticut King' were collected at each tepal developmental stage. Tepal segments about 7 mm long and 5 - 15 mm wide that did not include midribs were cut from basal or upper parts of tepals and were used for RNA isolation. The basal part segments in 'Montreux' contained tepal spots but the upper part segments did not. Similarly, tepals of field grown F_1 plants derived from crosses between 'Montreux' and 'Connecticut King' were collected at stage 2 or 5. To analyze organ-specific gene expression, anthers, filaments, styles, ovaries and tepals at tepal developmental stage 4 and leaves in field-grown 'Montreux' were collected.

To analyze the effect of light on gene expression in leaves, 'Vivaldi' bulbs were germinated in complete darkness for 2 weeks and then were grown in a 16 h-light/8 h-dark photoperiod at 23°C. Each leaf of light-treated plants was collected at 0, 2, 4, 6 and 8 d of light exposure and was used to isolate RNA (details in Nakatsuka et al. 2009).

RNA isolation and cDNA synthesis

Total RNA was isolated from 50 - 100 mg fresh weight materials and was purified using the RNeasy Plant Mini Kit combined with RNase-free DNase (Qiagen, Germany). First strand cDNA was synthesized using Super Script III reverse transcriptase (Invitrogen Japan) and oligo dT or poly T-adapter primers (Supplementary Table S1 lists all primer sequences).

Isolation of *R2R3-MYB* homologs from lily tepals

DNA fragments that encode R2R3-MYB genes were amplified by PCR with cDNA from ‘Montreux’ tepals (stage 4) and degenerate primers dMYBf and dMYBr that were originally designed from the conserved DNA-binding domain of R2R3-MYB proteins that control anthocyanin biosynthesis in other plant species. Amplified fragments were inserted into pGEM T-easy vector using the TA-cloning method (Promega Japan) and were sequenced using an ABI DNA sequencer and a BigDye Terminator Sequencing system (Applied Biosystems Japan). Full-length cDNA sequences were determined by RACE-PCR. 3´RACE-PCR was done between an adapter-1 primer and each gene specific primer. Single fragments were amplified by nested PCR between an adapter-1 primer and each nested primer. 5´RACE PCR was done as follows (Masuda et al. 1997). First strand cDNA of ‘Montreux’ tepals transcribed by oligo dT primer was treated with RNase H (Takara, Japan) and was purified using Microcon YM-100 centrifugal filter devices (Millipore Japan). Poly G tail was added to the 3’ terminal of the first strand cDNA using terminal deoxynucleotidyl transferase and dGTP (Roche, Switzerland). The plus strands were amplified using PrimeSTAR HD DNA polymerase (Takara) and poly C-adapter primer. After purifying the product using a High Pure PCR Product

Purification kit (Roche), the 5’ end sequence was amplified by PCR with an adapter-2 primer and each gene specific primer. Single fragments were amplified using an adapter-2 primer and each nested primer. After purifying the RACE-PCR products using a High Pure PCR Product Purification kit (Roche), they were sequenced directly using an ABI DNA sequencer and a BigDye Terminator Sequencing system (Applied Biosystems Japan).

The cDNA sequences were translated to amino acid sequences using Four Peaks software (http://mekentosj.com/4peaks/). Deduced amino acid sequences in lily genes and appropriate genes in other plant species (Fig. 2) were aligned using CLUSTALW (Thompson et al. 1994) at gap open penalty 10 and gap extension penalty 0.05. The genetic distances were calculated using Kimura 2-parameter (Kimura 1980), and a phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987). One hundred bootstrap replicates were analyzed. Software PROTDIST, NEIGHBOR, DRAWGRAM, SEQBOOT and CONSENSE from a PHYLIP 3.6 package (http://evolution.genetics.washington.edu/phylip.html) were used for the calculations.

Yeast two-hybrid analysis

In the yeast two-hybrid assay, the Matchmaker Two-Hybrid System 3 (Clontech, Mountain View, CA, USA) was employed to investigate protein interactions between LhbHLH2 (accession no. AB222076) and LhMYB6, and between LhbHLH2 and LhMYB12. The open reading frame (ORF) sequences of *LhMYB6*, *LhMYB12* and *LhbHLH2* were amplified by PCR and were cloned into the pGAD-T7 (*LhMYB6* and *LhMYB12*) or pGBK-T7 (*LhbHLH2*) vectors (Clontech). All constructs were transformed into *Saccharomyces cerevisiae* strain AH109 (Clontech) using the S.c.

easyComp transformation kit (Invitrogen Japan). Yeast transformants were grown at 30°C for 2 or 4 d on a selective medium without leucine (-LEU) and tryptophan (-TRP), on a selective medium without leucine, tryptophan, histidine (-HIS) and adenine (-ADE) or on a selective medium without leucine, tryptophan, histidine and adenine with 10 mM 3-AT.

Transient expression assay of LhMYB6 and LhMYB12

To construct vectors for a transient expression assay, *LhMYB6*, *LhMYB12* and *LhbHLH2* ORF fragments were amplified. Vectors p35Spro-LhMYB6, p35Spro-LhMYB12 and p35Spro-LhbHLH2 were constructed to replace the *enhanced β-glucuronidase* (*EGUS*) gene of pKD0330 with each ORF by using restriction sites *Stu*I and *Sma*I. The pKD0330 is a pBluescript vector containing the *EGUS* gene under the control of the *cauliflower mosaic virus* 35S promoter and a nopaline synthase terminator from *Agrobacterium tumefaciens* (K. Fujino, unpublished).

To evaluate whether *LhMYB6*, *LhMYB12* and *LhbHLH2* were responsible for regulation of anthocyanin biosynthesis in lily, transient expression assays were done using bulbscales of ‘Montreux’ and particle bombardment system PDS-1000/He (Bio-Rad, Hercules, CA, USA). One milligram of gold particles (1 μm in diameter) was precipitated together with 7.5 μg p35Spro-LhMYB6 or p35Spro-LhMYB12 plasmids and 15 μg p35Spro-LhbHLH2 plasmid, using $CaCl_2$ and spermidine, and was finally suspended in 22 μl ethanol. Pieces of sterilized bulbscales were placed on plates containing 20 ml of Linsmaier and Skoog nutrient solution supplemented with 1 % agar (Wako Pure Chemical, Japan) and were bombarded using 10 μl particles for each shot and 650 psi pressure plates. After 48 h of bombardment, RNA was isolated from the bulbscales and RT-PCR was done. Primer sets of LhMYB12cf - LhMYB12jr for

LhMYB12 and LhbHLH2cf - LhbHLH2dr for *LhbHLH2*, and primers for RT-PCR for *LhMYB6*, *LhDFR* (accession no. AB058641), *LhCHSa* (AB058638) and *LhCHSb* (AB058639) were used. Reaction conditions consisted of preheating at 94°C for 5 min, 32 cycles (*LhMYB6* and *LhCHSa*) or 35 cycles (*LhMYB12*, *LhbHLH2*, *LhDFR* and *LhCHSb*) at 94°C for 30 s and at 60°C for 80 s, and final extension at 72°C for 5 min.

Expression analysis of *LhMYB6* and *LhMYB12* in Asiatic hybrid lily

To investigate the temporal and spatial expressions of lily *MYB* genes and *LhDFR*, cDNA from flower organs and leaves and primer pairs for RT-PCR were used for real-time PCR. SYBR Premix Ex Taq (Takara) was used to intercalate SYBR Green I in amplified products. Signals were monitored using the Chromo4 real time PCR system (Bio-Rad Japan). Reaction conditions consisted of preheating at 94°C for 5 min, 40 cycles at 94°C for 30 s and at 63°C for 60 s (*LhMYB6*) or at 60°C for 90 s (*LhMYB12*, *LhbHLH2* and *LhDFR*), and final extension at 72°C for 5 min. The amount of *LhActin* mRNA (accession no. AB438963) in each sample was determined to normalize the differences in mRNA amount of other genes.

Supplementary data

Supplementary data are available at PCP online.

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Figure legends

Fig. 1 Tepal appearances of Asiatic hybrid lily cultivar ‘Montreux’ at tepal developmental stages 1 to 5. Both abaxial (left) and adaxial (right) sides of inner tepals at each developmental stage are shown. Note that spots became visible at stage 2 in the basal part of the adaxial side, and tepal pigmentation began at stage 3. Numerals indicate tepal developmental stages. Bar = 1 cm

Fig. 2 A neighbor-joining phylogenetic tree of plant R2R3-MYB sequences. Numbers next to the nodes are bootstrap values from 100 replications. The bar indicates a genetic distance of 0.1. The deduced amino acid sequences were retrieved from the DDBJ/EMBL/GenBank databases. AmMIXTA (CAA55725), Am305 (P81391), AmROSEA1 (ABB83826) and AmVENOSA (ABB83828) in *Antirrhinum majus*, AtMYB16 (NP_197035), AtMYB24 (AAM63674), AtMYB33 (NP_850779), AtTT2 (NP_198405), AtPAP1 (NP_176057) and AtPAP2 (NP_176813) in *Arabidopsis*, HvGAMYB (X87690) in *Hordeum vulgare*, OgMYB1 (ABS58501) in *Oncidium* Gower Ramsey, PhMyb9 (ACH95795) in *Phalaenopsis* hybrid, LjTT2a (BAG12893), LjTT2b (BAG12894) and LjTT2c (BAG12895) in *Lotus japonicus*, ZmPl (AAA19821) and ZmC1 (P10290) in *Zea mays*, MdMYB10a (ABB84753) in *Malus* x *domestica*, GhMYB10 (CAD87010) in *Gerbera hybrida*, VlMYBA1-1 (BAC07537) in *Vitis labrusca* x *V. vinifera*, MaMYB (ACA04006) in *Mimulus aurantiacus*, InMYB2 (BAE94709) in *Ipomoea nil*, and PhAN2 (AAF66727) in *Petunia* x *hybrida*.

Fig. 3 Yeast two-hybrid analysis to examine the protein-protein interaction between LhbHLH2 and LhMYB6, and LhbHLH2 and LhMYB12. LhbHLH2 protein was fused

to the GAL4 DNA-binding domain and was assayed for its ability to bind the LhMYB6 or LhMYB12 fused to the GAL4 activation domain. Interaction is shown by yeast growth on a quadruple dropout medium supplemented with or without 10 mM 3-AT.

Fig. 4 Effect of transient expression of *LhMYB6* and *LhMYB12* genes together with or without *LhbHLH2* gene on *LhDFR*, *LhCHSa* and *LhCHSb* transcription in lily bulbscales. 48 h after bombardment, transcript accumulation of *LhbHLH2*, *LhMYB6*, *LhMYB12*, *LhDFR*, *LhCHSa* and *LhCHSb* were determined by RT-PCR.

Fig. 5 Temporal and spatial expression of *LhMYB6* (A and D), *LhMYB12* (B and E) and *LhDFR* (C and F). A, B and C: Relative expression in flower tepals (developmental stages 1 to 5) of Asiatic hybrid lily cultivars 'Montreux' (basal and upper parts) and 'Connecticut King' (basal part). Transcription of *LhMYB12* and *LhDFR* was not detected in tepals of 'Connecticut King'. D, E and F: Relative expression in five flower organs collected from stage 4 flowers and in leaves of 'Montreux'. *LhActin* was used to normalize the expression of these genes. Vertical bars indicate the standard error (n=3).

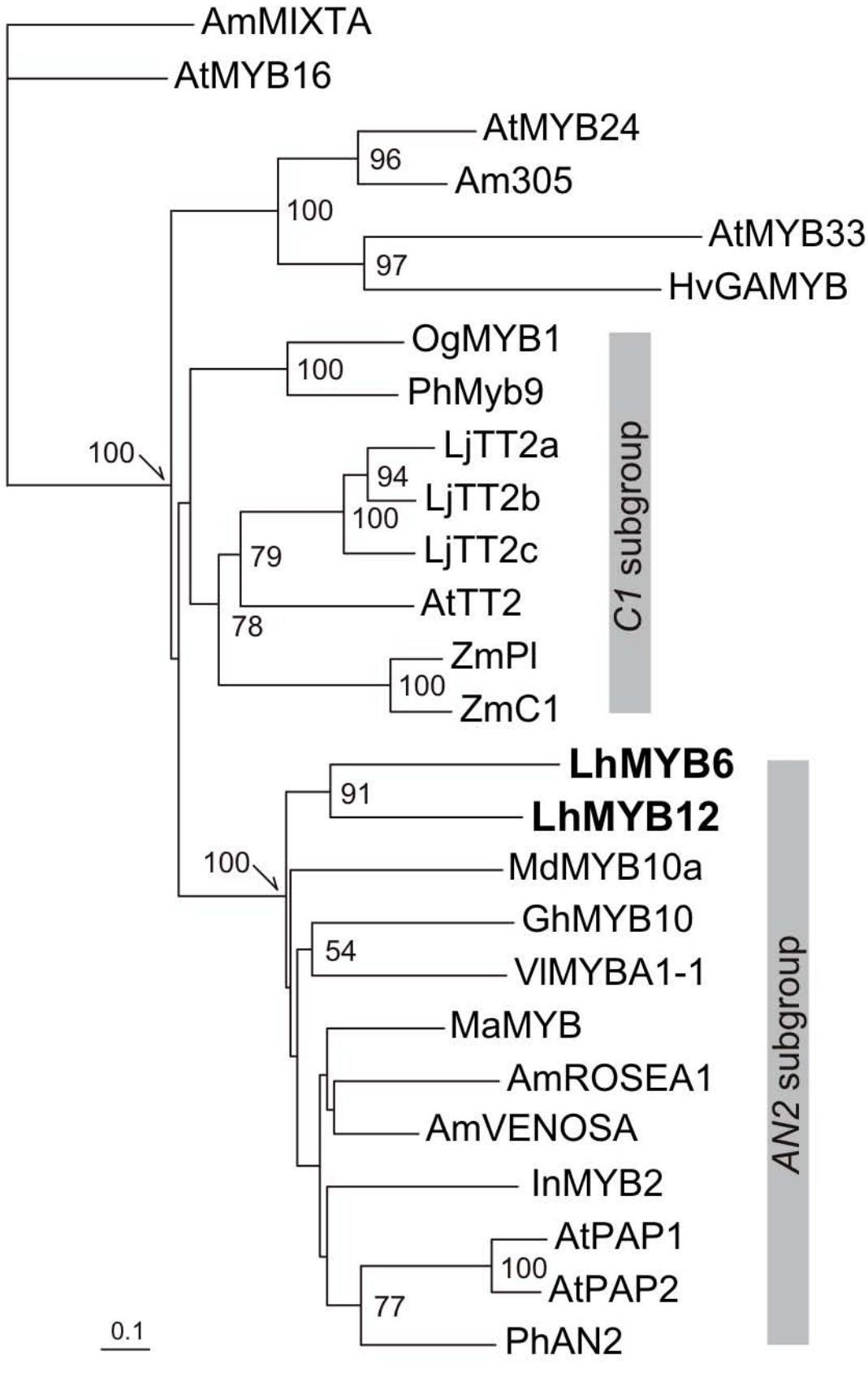
Fig. 6 Relative expression of *LhMYB6* in tepals of 'Montreux' (Mon) (n=2), 'Connecticut King' (CK) (n=2), yellow tepal F_1 plants with tepal spots (n=4) and pink tepal F_1 plants without tepal spots (n=4). F_1 plants were derived from crosses between 'Montreux' and 'Connecticut King'. Stage 2 tepals were used in this analysis. *LhActin* was used to normalize the expression of the gene. Vertical bars indicate the standard error.

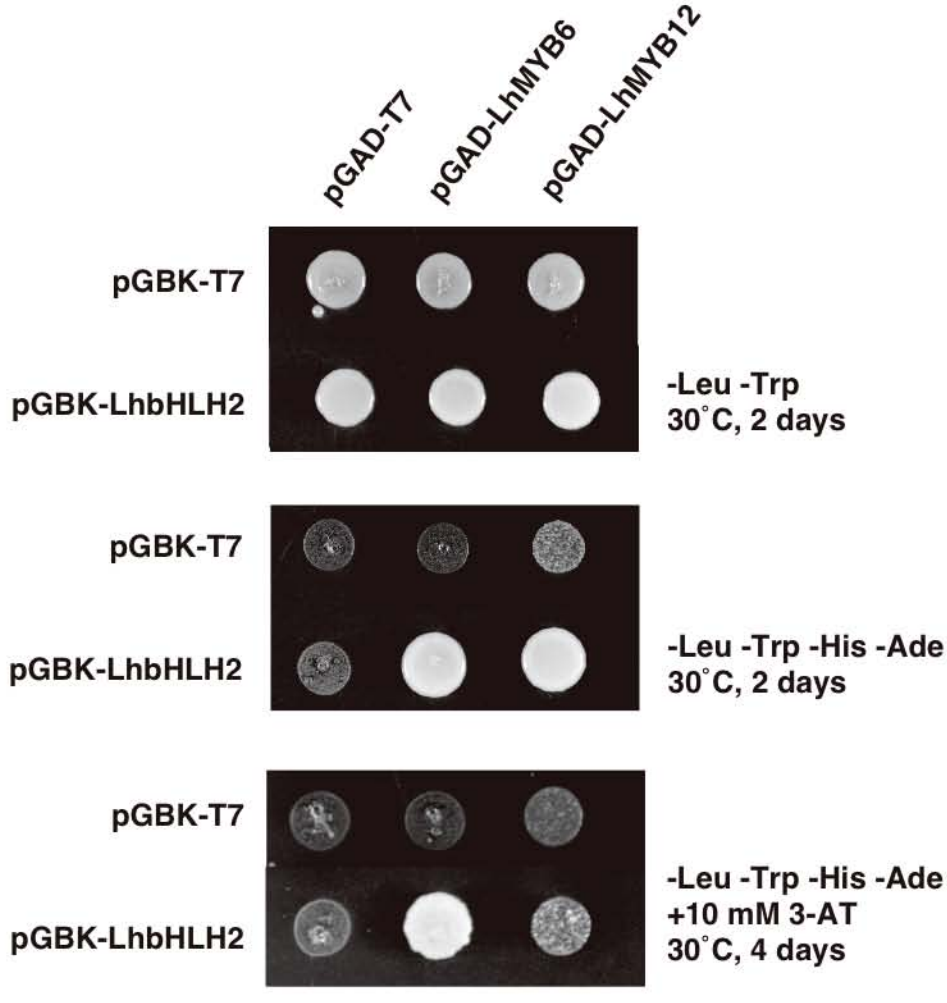
Fig. 7 SYBR Green I-stained PCR products of *LhMYB12* in tepals of 'Montreux' (Mon),

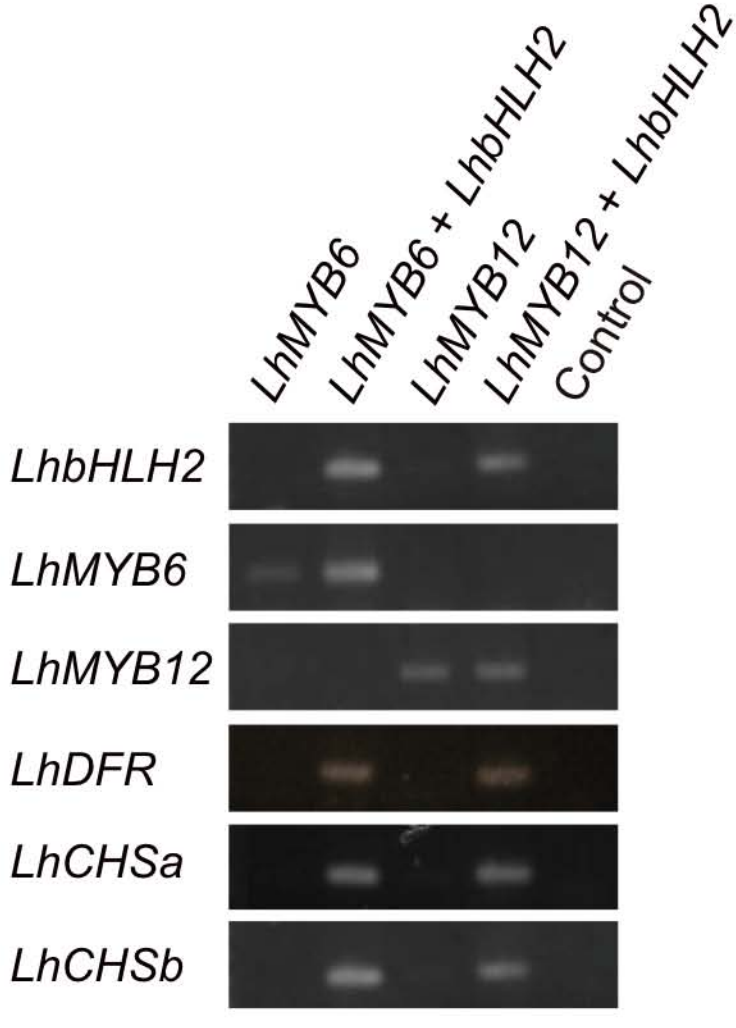
‘Connecticut King’ (CK), F_1 plants having yellow or white tepals (no anthocyanin) and F_1 plants having pink tepals (by anthocyanin). Stage 5 tepals were used in this experiment. F_1 plants were derived from ‘Montreux’ and ‘Connecticut King’ crosses.

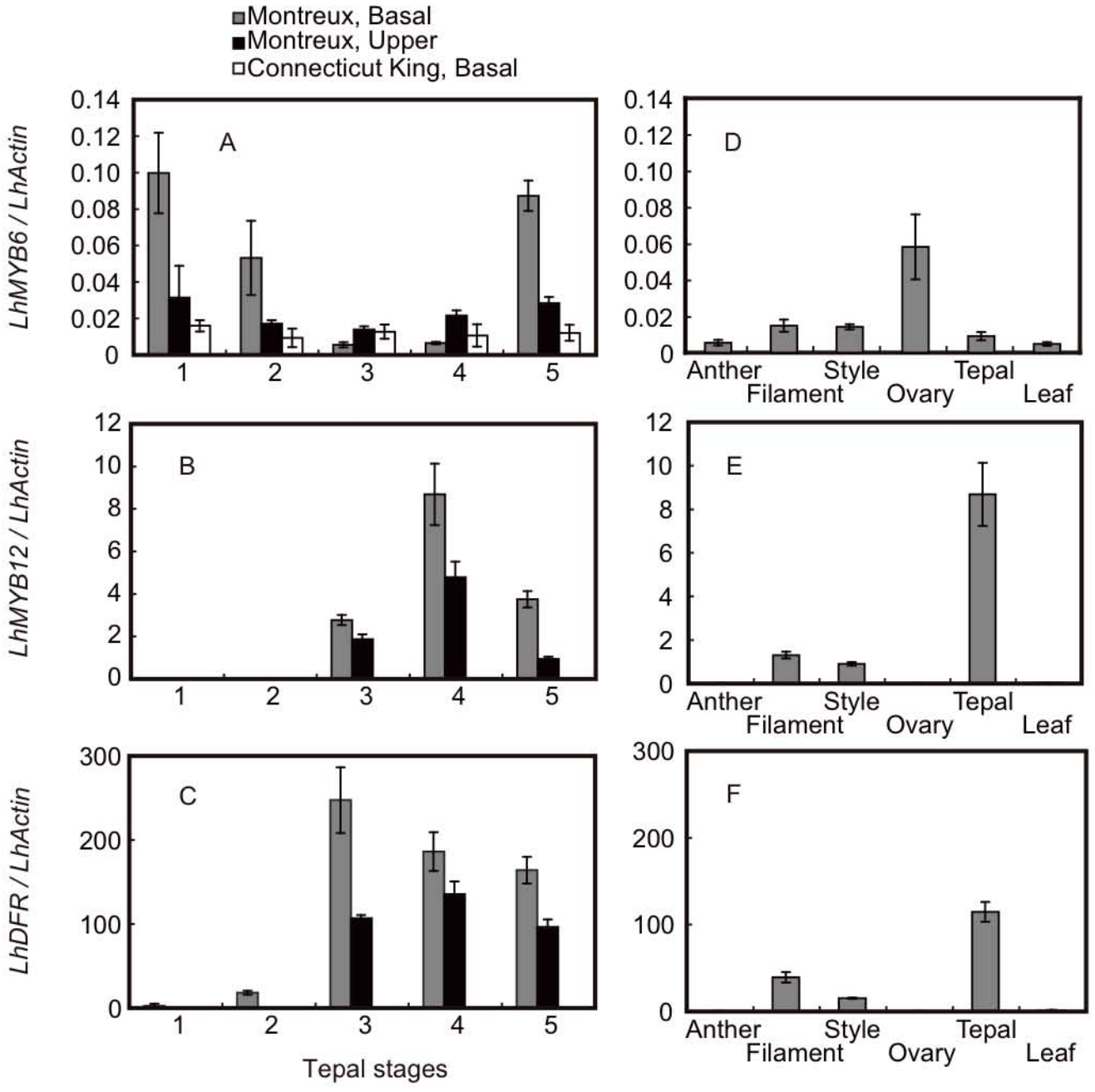
Fig. 8 Effect of light exposure on relative expression of *LhMYB6* (A) and *LhDFR* (B) in leaves of Asiatic hybrid lily cultivar ‘Vivaldi’. Bulbs were germinated in the dark for 2 weeks, and then were grown in a 16h-light/8h-dark photoperiod for 0, 2, 4, 6 and 8 d. *LhActin* was used to normalize the expression of the gene. Vertical bars are the standard error (n=3).

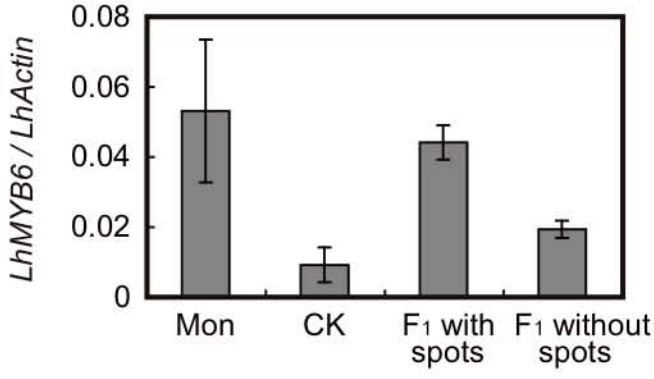

1 cm
1
2
3
4
5

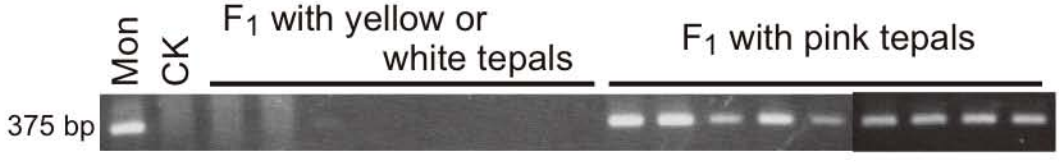

AmMIXTA
AtMYB16
AtMYB24
96
Am305
100
AtMYB33
97
HvGAMYB
OgMYB1
100
PhMyb9
100
LjTT2a
94
LjTT2b
100
LjTT2c
79
AtTT2
78
ZmPl
100
ZmC1
C1 subgroup
LhMYB6
91
LhMYB12
100
MdMYB10a
GhMYB10
54
VlMYBA1-1
MaMYB
AmROSEA1
AmVENOSA
InMYB2
AtPAP1
100
AtPAP2
77
PhAN2
AN2 subgroup
0.1

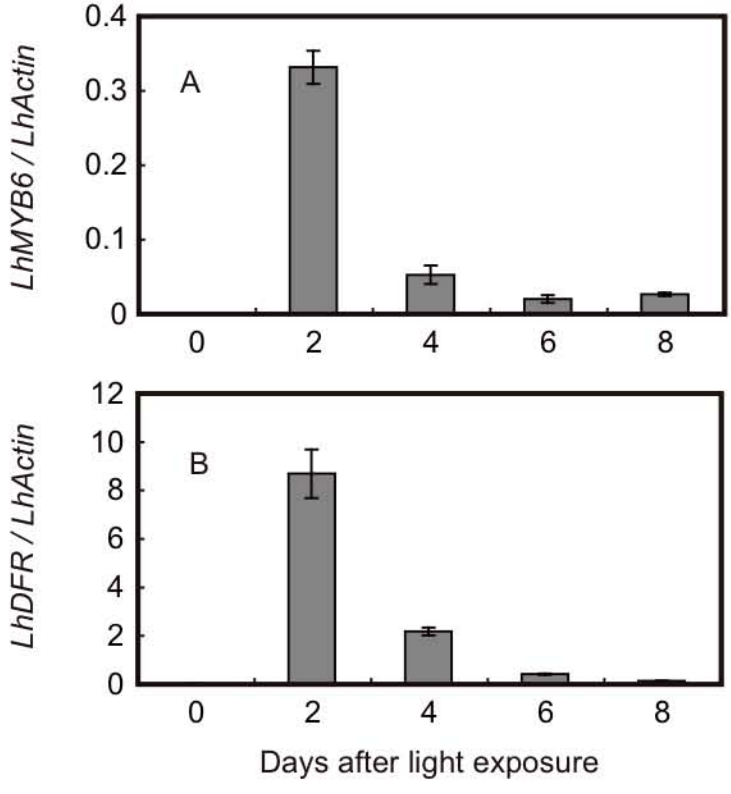

pGAD-T7
pGAD-LhMYB6
pGAD-LhMYB12
pGBK-T7
pGBK-LhbHLH2
-Leu -Trp
30˚C, 2 days
pGBK-T7
pGBK-LhbHLH2
-Leu -Trp -His -Ade
30˚C, 2 days
pGBK-T7
pGBK-LhbHLH2
-Leu -Trp -His -Ade
+10 mM 3-AT
30˚C, 4 days


LhMYB6
LhMYB6 + LhbHLH2
LhMYB12
LhMYB12 + LhbHLH2
Control
LhbHLH2
LhMYB6
LhMYB12
LhDFR
LhCHSa
LhCHSb


Montreux, Basal
Montreux, Upper
Connecticut King, Basal
LhMYB6 / LhActin
LhMYB12 / LhActin
LhDFR / LhActin
A
B
C
D
E
F
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
12
10
8
6
4
2
300
200
100
1
2
3
4
5
Tepal stages
Anther
Filament
Style
Ovary
Tepal
Leaf


0.08
0.06
0.04
0.02
0
LhMYB6 / LhActin
Mon
CK
F1 with spots
F1 without spots


Mon
CK
F1 with yellow or white tepals
F1 with pink tepals
375 bp


0.4
0.3
0.2
0.1
0
A
LhMYB6 / LhActin
0
2
4
6
8
12
10
8
6
4
2
0
B
LhDFR / LhActin
0
2
4
6
8
Days after light exposure